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**PROVISIONAL APPLICATION FOR PATENT
COVER SHEET**

Case No. **NIH272.002PR**

Date: February 4, 2004

Page 1

Commissioner for Patents
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ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **IDENTIFICATION OF CHIMPANZEE FAB FRAGMENTS BY REPERTOIRE
CLONING AND PRODUCTION OF A FULL-LENGTH HUMANIZED IGG1
ANTIBODY HIGHLY EFFICIENT FOR NEUTRALIZATION OF DENGUE TYPE 4
VIRUS**

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Enclosed are:

- (X) Specification in 54 pages.
- (X) 7 sheets of drawings.
- (X) A check in the amount of \$160 to cover the filing fee is enclosed.
- (X) A return prepaid postcard.
- (X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410.

Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

- (X) Yes. The name of the U.S. Government agency and the Government contract number are: National Institutes of Health.
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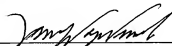
**PROVISIONAL APPLICATION FOR PATENT
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Case No. NIH272.002PR

Date: February 4, 2004

Page 2

Respectfully submitted,



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MAIL STOP PROVISIONAL PATENT APPLICATION

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Attorney Docket No. : NIH272.002PR

Applicant(s) : Lai et al.

For : IDENTIFICATION OF CHIMPANZEE FAB
FRAGMENTS BY REPERTOIRE CLONING AND
PRODUCTION OF A FULL-LENGTH
HUMANIZED IGG1 ANTIBODY HIGHLY
EFFICIENT FOR NEUTRALIZATION OF
DENGUE TYPE 4 VIRUS

Attorney : Nancy W. Vensko

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Date of Deposit : February 4, 2004

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Transmittal letter; specification in 54 pages; 7 sheets of drawings; Check for
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**IDENTIFICATION OF CHIMPANZEE FAB FRAGMENTS BY REPERTOIRE CLONING
AND PRODUCTION OF A FULL-LENGTH HUMANIZED IGG1 ANTIBODY HIGHLY
EFFICIENT FOR NEUTRALIZATION OF DENGUE TYPE 4 VIRUS**

Field of the Invention

This invention relates generally to the field of immunology and specifically to monoclonal antibodies that bind or neutralize dengue type 4 virus.

Background of the Invention

Among the arthropod-borne flaviviruses, the four dengue virus serotypes (DENV-1-4) that constitute a serologically distinct subgroup are most important in terms of human morbidity and geographic distribution. Dengue viruses cause dengue outbreaks and major epidemics in most tropical and subtropical areas where *Aedes albopictus* and *Aedes aegypti* mosquitos are abundant. Dengue infection produces fever, rash, and joint pain in humans. A more severe and life-threatening form of dengue, characterized by hemorrhagic fever and hemorrhagic shock, has occurred with increasing frequency in Southeast Asia and Central and South America, where all four dengue virus serotypes circulate. The underlying cause of severe dengue remains controversial (Halstead, S. 1982 *Progress in Allergy*. 31:301-364; Rosen, L 1986 *Am. J. Trop. Med. Hyg.* 35:642- 653). An association of severe dengue with increased viral replication has been reported recently (Wang, W. K. *et al.* 2002 *J. Virol.* 76:4662- 4665). A safe and effective vaccine against dengue is currently not available.

The dengue virus contains a positive strand RNA genome, coding for a polyprotein that is cleaved co- and post-translationally by a combination of cellular and viral proteases to generate the individual viral proteins (Markoff, L. 1989 *J. Virol.* 63:3345-3352; Chambers, T. J. *et al.* 1990. *Ann. Rev. Microbiol.* 44:649-688; Falgout, B. *et al.* 1991 *J. Virol.* 65:2467-2475). Dengue virus prM and E structural proteins and nonstructural NS1 protein are glycosylated. The prM glycoprotein is further cleaved by the cellular enzyme furin following viral assembly, generating M, which is present in the mature virus (Stadler, K. *et al.* 1997 *J. Virol.* 71:8475-8481). Flavivirus prM and E form heterodimers, which are assembled into viral particles during infection (Wengler, G. and G. Wengler 1989 *J. Virol.* 63:2521-2526). In this manner, the prM serves to protect the functional integrity of E from acid-induced conformational change (Heinz, F. X. *et al.* 1994 *Virology* 198:109-117; Holzmann, H. *et al.*

1995 *Arch. Virol.* **140**:213-221). The E glycoprotein is responsible for cell attachment, possibly mediated by a receptor, and for fusion with the cell membranes following viral entry.

Mouse monoclonal antibodies against the dengue viruses have been valuable for dengue virus serotype determination (Gentry, M. K. *et al.* 1982 *Am. J. Trop. Med. Hyg.* **31**:548-555; Henchal, E. A. *et al.* 1982 *Am. J. Trop. Med. Hyg.* **31**:830-836). Studies using monoclonal antibodies against dengue and other flaviviruses have also provided valuable information concerning the antigenic structure of the major viral antigen E (Heinz, F. X. *et al.* 1983 *Virology* **126**:525-537; Henchal, E. A. *et al.* 1985 *Am. J. Trop. Med. Hyg.* **34**:162-169; Heinz, F. X. 1986 *Adv. Virus Res.* **31**:103-168; Mandl, C. W. *et al.* 1989 *J. Virol.* **63**:564-571; Roehrig, J. T. *et al.* 1998 *Virology* **246**:317-328). The 3-dimensional structure of the E glycoprotein has been determined at 2 Å resolution for tick-borne encephalitis virus and recently for dengue type 2 virus (Rey, P. A. *et al.* 1995 *Nature* **375**:291-298; Modis, Y. *et al.* 2003 *Proc. Natl. Acad. Sci. USA* **100**:6986-6991). These studies showed that the monomeric E polypeptide is folded into three distinct domains and that the E glycoprotein consists of a flat elongated dimer structure with an inter-domain ligand-binding pocket.

Monoclonal antibodies reactive to flavivirus envelope proteins have been shown to mediate protection against homologous virus challenge in animal models (Mathews, J. H. and J. T. Roehrig 1984 *J. Immunol.* **132**:1533-1537; Brandriss, M. W. *et al.* 1986 *J. Gen. Virol.* **67**:229-234; Gould, E. A. *et al.* 1986 *J. Gen. Virol.* **67**:591-595; Kaufman, B.M. *et al.* 1987 *Am. J. Trop. Med. Hyg.* **36**:427-434; Kimura-Kuroda, J., and K. Yasui 1988 *J. Virol.* **141**:3606-3610). In most cases, protection by passive immunization has been correlated with the ability of these antibodies to neutralize the virus *in vitro*. Protection against dengue virus challenge was also demonstrated in mice following passive immunization with monoclonal or polyclonal antibodies specific to prM (Bray, M., and C. J. Lai. 1991 *Virology* **185**:505-508; Kaufman, BM *et al.* 1987 *Am. J. Trop. Med. Hyg.* **36**:427-434) or NS1 (Falgout, B. *et al.* 1990. *J. Virol.* **64**:4356-4363; Henchal, E. A. *et al.* 1988 *J. Gen. Virol.* **69**:2101-2107).

Most research efforts directed to the development of an attenuated live dengue vaccine have not yielded a satisfactory result. Recently, clinical evaluation was conducted on a genetically engineered DENV-4 mutant containing a 30-nucleotide deletion in the 3' non-

coding region that exhibited reduced replicative capacity in simian cell culture and in primates (Durbin, A. P. *et al.* 2001 *Am. J. Trop. Med. Hyg.* **65**:405-413; Men R., *et al.* 1996 *J. Virol.* **70**:3930-3937). Following a single dose inoculation, a total of 20 volunteers remained afebrile and exhibited very few clinical signs. Each of the vaccinees developed a high titer of DENV-4 neutralizing antibodies four to six weeks after immunization. However, five vaccinees showed an elevation of serum levels of the liver enzyme alanine transaminase (ALT). The ALT elevations were mostly transient and eventually subsided, but there remains a concern about the safety of a live dengue virus vaccine. Passive immunization with clinically acceptable dengue virus neutralizing antibodies provides an attractive alternative to prevention of dengue virus infection. Highly efficient neutralizing antibodies might also be useful for consideration as a possible therapy for severe dengue. Recently, phage display of combinatorial antibody libraries has allowed isolation of antibodies against important viral pathogens from human or non-human primates (Persson, M. A. *et al.* 1991 *Proc. Natl. Acad. Sci.* **88**:2432-2436; Williamson, R. A. *et al.* 1993 *Proc. Nat. Acad. Sci.* **90**:41413-4145 [Erratum **91**:1193, 1994]; Burton, D. R. *et al.* 1994 *Science* **266**:1024-1027; Crowe, J. E. Jr. *et al.* 1994. *Proc. Natl. Acad. Sci.* **91**:1386-1390; Maruyama, T. *et al.* 1999 *J. Virol.* **73**:6024-6030; Schofield, D. J. *et al.* 2000 *J. Virol.* **74**:5548-5555).

Segue to the Invention

In the current study, we employed this technique to identify a panel of chimpanzee Fab antibodies against DENV-4. One of these Fab antibodies neutralized DENV-4 efficiently by an *in vitro* assay and was combined with human sequences to convert it to the whole IgG1 antibody. The humanized chimpanzee IgG1 antibody produced in CHO cells neutralized DENV-4 efficiently.

Summary of the Invention

The present invention relates to chimpanzee Fab antibody fragments obtained by repertoire cloing and their derived humanized monoclonal antibodies that bind or neutralize dengen type 4 virus. The invention provides such antibody fragments, such humanized or chimeric antibodies, and pharmaceutical compositions including such antibodies. The invention further provides for isolated nucleic acids encoding the antibodies of the invention and host cells transformed therewith. Additionally, the invention provides for prophylactic,

therapeutic, and diagnostic methods employing the antibodies and nucleic acids of the invention.

Brief Description of the Drawings

Figure 1. A map of pFab CMV-dhfr vector for expression of full-length IgG1 in CHO cells and structure of the IgG1 light chain and heavy chain DNA inserts. (A) Locations of the various genes present in the expression vector- *dhfr*, dihydro-reductase; *neo*, neomycin phosphotransferase; hCMV, human CMV promoter; LC, light chain DNA; pA, polyA addition signal; *amp*, pBR 322 β -lactamase and the DNA replication origin; HC, heavy chain DNA. Arrows indicate transcription direction. (B) Structure of the humanized IgG1 light chain and heavy chain genes under the control of a hCMV early promoter. V_L and C_L are the light chain hyper-variable region and constant region, respectively; V_H , C_H1 , hg, int-1, C_H2 , int-2, and C_H3 represent the heavy chain hyper-variable region, constant region 1, hinge, intron-1 (118 nucleotides), constant region 2, intron-2 (97 nucleotides) and constant region 3 in that order. The dark-shaded regions are human IgG1 sequences and the medium-shaded regions represent chimpanzee IgG1 sequences. The selectable *neo* and *dhfr* genes (light-shaded) are flanked by a β -globin promoter and a poly A addition site.

Figure 2. Alignment of amino acid sequences among DENV-4-specific Fab monoclonal antibodies. The amino acid sequences of the six chimpanzee Fab monoclonal antibodies recovered by repertoire cloning were compared. (A) Sequences of V_L light chain segments. (B) V_H heavy chain segments. The framework regions (FR1-4) and complementarity-determining regions (CDR1-3) are shown. The dash symbol is placed where an amino acid deletion occurred and an identical amino acid is represented by a comma.

Figure 3. Analysis of antigenic specificity by radio-immunoprecipitation. (A) ^{35}S -methionine labeled lysates of DENV-4-infected Vero cells were precipitated with the various Fab preparations indicated. (B) ^{35}S -methionine labeled lysates were prepared from Vero cells infected with vaccinia virus recombinant vDENV-4 prM or vDENV- E containing the full length coding sequence of prM or E, respectively. E+prM: precipitations using a mixture of both lysates. HMAF: precipitation using hyper-immune mouse ascitic fluid raised against DENV-4.

Figure 4. Epitope analysis of chimpanzee Fab antibodies against DENV-4 by competition ELISA. Selected Fabs were affinity purified, biotinylated and used for analysis of binding reactivity to DENV-4 virions by competition ELISA in the presence of competing, unlabeled Fabs. (A) Biotinylated Fab 3C1; (B) Biotinylated Fab 3E4; (C) Biotinylated Fab 7G4; (D) Biotinylated Fab 5H2. Chimpanzee Fab 1F2, which did not bind to DENV-4, was used as a negative control. The numbers on the Y-axis are OD readings and the X-coordinate represents reciprocal dilutions of the competing Fabs.

Figure 5. *In vitro* neutralization of DENV-4 strains by humanized chimpanzee antibody IgG1 5H2. Full-length antibody IgG1 5H2 was concentrated from the culture medium of transformed CHO cells selected with 2×10^{-7} M methotrexate and then affinity-purified through a protein-A column. The neutralizing activity of the antibody preparation was tested by PRNT against DENV-4 H241 isolated in the Philippines and DENV-4 814669 and DEV-4 341750, isolated in the Caribbean.

Brief Description of the SEQ. ID. NOs.

| Region | Light Chain 5A7 Sequence | Heavy Chain 5A7 Sequence | Light Chain 3C1 Sequence | Heavy Chain 3C1 Sequence | Light Chain 3E4 Sequence | Heavy Chain 3E4 Sequence | Light Chain 7G4 Sequence | Heavy Chain 7G4 Sequence | Light Chain 5H2 Sequence | Heavy Chain 5H2 Sequence | Light Chain 5D9 Sequence | Heavy Chain 5D9 Sequence |
|--------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| FR1 | SEQ. ID. NO: 1 | SEQ. ID. NO: 8 | SEQ. ID. NO: 15 | SEQ. ID. NO: 22 | SEQ. ID. NO: 29 | SEQ. ID. NO: 36 | SEQ. ID. NO: 43 | SEQ. ID. NO: 50 | SEQ. ID. NO: 57 | SEQ. ID. NO: 64 | SEQ. ID. NO: 71 | SEQ. ID. NO: 78 |
| CDR1 | SEQ. ID. NO: 2 | SEQ. ID. NO: 9 | SEQ. ID. NO: 16 | SEQ. ID. NO: 23 | SEQ. ID. NO: 30 | SEQ. ID. NO: 37 | SEQ. ID. NO: 44 | SEQ. ID. NO: 51 | SEQ. ID. NO: 58 | SEQ. ID. NO: 65 | SEQ. ID. NO: 72 | SEQ. ID. NO: 79 |
| FR2 | SEQ. ID. NO: 3 | SEQ. ID. NO: 10 | SEQ. ID. NO: 17 | SEQ. ID. NO: 24 | SEQ. ID. NO: 31 | SEQ. ID. NO: 38 | SEQ. ID. NO: 45 | SEQ. ID. NO: 52 | SEQ. ID. NO: 59 | SEQ. ID. NO: 66 | SEQ. ID. NO: 73 | SEQ. ID. NO: 80 |
| CDR2 | SEQ. ID. NO: 4 | SEQ. ID. NO: 11 | SEQ. ID. NO: 18 | SEQ. ID. NO: 25 | SEQ. ID. NO: 32 | SEQ. ID. NO: 39 | SEQ. ID. NO: 46 | SEQ. ID. NO: 53 | SEQ. ID. NO: 60 | SEQ. ID. NO: 67 | SEQ. ID. NO: 74 | SEQ. ID. NO: 81 |
| FR3 | SEQ. ID. NO: 5 | SEQ. ID. NO: 12 | SEQ. ID. NO: 19 | SEQ. ID. NO: 26 | SEQ. ID. NO: 33 | SEQ. ID. NO: 40 | SEQ. ID. NO: 47 | SEQ. ID. NO: 54 | SEQ. ID. NO: 61 | SEQ. ID. NO: 68 | SEQ. ID. NO: 75 | SEQ. ID. NO: 82 |
| CDR3 | SEQ. ID. NO: 6 | SEQ. ID. NO: 13 | SEQ. ID. NO: 20 | SEQ. ID. NO: 27 | SEQ. ID. NO: 34 | SEQ. ID. NO: 41 | SEQ. ID. NO: 48 | SEQ. ID. NO: 55 | SEQ. ID. NO: 62 | SEQ. ID. NO: 69 | SEQ. ID. NO: 76 | SEQ. ID. NO: 83 |
| FR4 | SEQ. ID. NO: 7 | SEQ. ID. NO: 14 | SEQ. ID. NO: 21 | SEQ. ID. NO: 28 | SEQ. ID. NO: 35 | SEQ. ID. NO: 42 | SEQ. ID. NO: 49 | SEQ. ID. NO: 56 | SEQ. ID. NO: 63 | SEQ. ID. NO: 70 | SEQ. ID. NO: 77 | SEQ. ID. NO: 84 |

Deposit of Biological Material

The following biological material has been deposited in accordance with the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), Manassas, VA, on the date indicated:

| Biological material | Designation No. | Date |
|-----------------------------|-----------------|-------------------|
| Plasmid: Humanized IgG1 5H2 | PTA-5662 | November 26, 2003 |

The Plasmid: Humanized IgG1 5H2 was deposited as ATCC Accession No. PTA-5662 on November 26, 2003 with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, USA. This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicant and ATCC which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC § 122 and the Commissioner's rules pursuant thereto (including 37 CFR § 1.14). Availability of the deposited biological material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Detailed Description of the Preferred Embodiment

A safe and effective dengue vaccine is still not available. Passive immunization using monoclonal antibodies from humans or non-human primates represents an attractive alternative for prevention of dengue. Fab monoclonal antibodies to dengue type 4 virus (DENV-4) were recovered by repertoire cloning of bone marrow mRNAs from an immune chimpanzee and analyzed for antigen binding specificity. Fabs 5A7, 3C1, 3E4 and 7G4 were

isolated from a library constructed from a chimpanzee following intra-hepatic transfection with infectious DENV-4 RNA. The V_H and V_L sequences and neutralizing activity against DENV-4 *in vitro* of these Fabs were analyzed. Fabs 5H2 and 5D9, which shared a nearly identical V_H sequence, but varied in their V_L sequences, were recovered from a library constructed from the same chimpanzee after super-infection with a mixture of DENV-1, DENV-2 and DENV-3 viruses. In radio-immunoprecipitation, Fab 5A7 precipitated only DENV-4 prM and Fabs 3E4, 7G4, 5D9 and 5H2 precipitated DENV-4 E but little or no prM. Fab 3E4 and Fab 7G4 competed with each other for binding to DENV-4 in ELISA, as did Fab 3C1 and Fab 5A7. Fab 5H2 recognized an epitope on DENV-4 that was separate from the epitope(s) recognized by other Fabs. Both Fab 5H2 and Fab 5D9 neutralized DENV-4 efficiently with a titer of 0.24-0.58 $\mu\text{g/ml}$ by plaque reduction neutralization test (PRNT), whereas DENV-4 neutralizing activity of other Fabs was low or not detected. Fab 5H2 was converted to full-length IgG1 by combining it with human sequences. The humanized chimpanzee antibody IgG1 5H2 produced in CHO cells neutralized DENV-4 strains from different geographical origins at a similar PRNT₅₀ titer of 0.03-0.05 $\mu\text{g/ml}$. The DENV-4 binding affinities were 0.42 nM for Fab 5H2 and 0.24 nM for full-length IgG1 5H2. Monoclonal antibody IgG1 5H2 is predicted to be invaluable for passive immunoprophylaxis against dengue in humans.

Definitions

As used herein, the term "antibody" means an immunoglobulin molecule or a fragment of an immunoglobulin molecule having the ability to specifically bind to a particular antigen. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only full-length antibody molecules but also fragments of antibody molecules retaining antigen binding and functional ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only full-length immunoglobulin molecules but also antigen binding active fragments such as the well-known active fragments $F(ab')_2$, Fab, Fv, and Fd.

As used herein, the term "dengue virus disease" means any disease caused, directly or indirectly, by one of the four serotypes of a dengue virus, which is a flavivirus. Dengue is an

acute febrile disease characterized by sudden onset, with headache, fever, prostration, joint and muscle pain, lymphadenopathy, and a rash that appears simultaneously with a temperature rise. A second phase of temperature rise may appear following an afebrile period. Dengue hemorrhagic fever/dengue shock syndrome is an acute disease occurring primarily in children characterized by an abrupt febrile onset followed by hemorrhagic manifestations and circulatory collapse.

As used herein with respect to polypeptides, the term "substantially pure" means that the polypeptides are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. In particular, the polypeptides are sufficiently pure and are sufficiently free from other biological constituents of their hosts cells so as to be useful in, for example, generating antibodies, sequencing, or producing pharmaceutical preparations. By techniques well known in the art, substantially pure polypeptides may be produced in light of the nucleic acid and amino acid sequences disclosed herein. Because a substantially purified polypeptide of the invention may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the polypeptide may comprise only a certain percentage by weight of the preparation. The polypeptide is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems.

As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated,

however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art.

As used herein, a coding sequence and regulatory sequences are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences, as desired.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids and phagemids. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such

that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification and selection of cells which have been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., B-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques. Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

Novel Anti-DENV-4 Monoclonal Antibodies

The present invention derives, in part, from the isolation and characterization of a novel chimpanzee Fab fragment and its humanized monoclonal antibody that selectively binds and neutralizes dengue type 4 virus and that we have designated 5H2. As described more fully herein, this new monoclonal antibody has been shown to bind and neutralize the dengue type 4 virus. The paratope of the 5H2 Fab fragment associated with the neutralization epitope on the dengue type 4 virus is defined by the amino acid (aa) sequences of the immunoglobulin heavy and light chain V-regions depicted in Fig. 2 and SEQ. ID. NO: 57 (FR1), SEQ. ID. NO: 58 (CDR1), SEQ. ID. NO: 59 (FR2), SEQ. ID. NO: 60 (CDR2), SEQ. ID. NO: 61 (FR3), SEQ. ID. NO: 62 (CDR3), and SEQ. ID. NO: 63 (FR4), and SEQ. ID. NO: 64 (FR1), SEQ. ID. NO: 65 (CDR1), SEQ. ID. NO: 66 (FR2), SEQ. ID. NO: 67 (CDR2), SEQ. ID. NO: 68 (FR3), SEQ. ID. NO: 69 (CDR3), and SEQ. ID. NO: 70 (FR4). The nucleic acid sequences coding for these aa sequences were identified as described in Example 1, by sequencing the Fab heavy chain and light chain fragments. Due to the

degeneracy of the DNA code, the paratope is more properly defined by the derived aa sequences depicted in Fig. 2.

The present invention derives, additionally in part, from the isolation and characterization of novel chimpanzee Fab monoclonal antibodies that selectively bind and precipitate dengue type 4 virus prM or E glycoproteins that we have designated 5A7, 3C1, 3E4, 7G4, 5H2, and 5D9. As described more fully herein, these new monoclonal antibodies have been shown to bind and precipitate the dengue type 4 virus prM or E glycoproteins. The paratopes of the 5A7, 3C1, 3E4, 7G4, 5H2, and 5D9 Fab fragment associated with the epitopes on the dengue type 4 virus is defined by the amino acid (aa) sequences of the immunoglobulin heavy and light chain V-regions depicted in Fig. 2 and, for 5A7, SEQ. ID. NO: 1 (FR1), SEQ. ID. NO: 2 (CDR1), SEQ. ID. NO: 3 (FR2), SEQ. ID. NO: 4 (CDR2), SEQ. ID. NO: 5 (FR3), SEQ. ID. NO: 6 (CDR3), and SEQ. ID. NO: 7 (FR4), and SEQ. ID. NO: 8 (FR1), SEQ. ID. NO: 9 (CDR1), SEQ. ID. NO: 10 (FR2), SEQ. ID. NO: 11 (CDR2), SEQ. ID. NO: 12 (FR3), SEQ. ID. NO: 13 (CDR3), and SEQ. ID. NO: 14 (FR4);

for 3C1, SEQ. ID. NO: 15 (FR1), SEQ. ID. NO: 16 (CDR1), SEQ. ID. NO: 17 (FR2), SEQ. ID. NO: 18 (CDR2), SEQ. ID. NO: 19 (FR3), SEQ. ID. NO: 20 (CDR3), and SEQ. ID. NO: 21 (FR4), and SEQ. ID. NO: 22 (FR1), SEQ. ID. NO: 23 (CDR1), SEQ. ID. NO: 24 (FR2), SEQ. ID. NO: 25 (CDR2), SEQ. ID. NO: 26 (FR3), SEQ. ID. NO: 27 (CDR3), and SEQ. ID. NO: 28 (FR4);

for 3E4, SEQ. ID. NO: 29 (FR1), SEQ. ID. NO: 30 (CDR1), SEQ. ID. NO: 31 (FR2), SEQ. ID. NO: 32 (CDR2), SEQ. ID. NO: 33 (FR3), SEQ. ID. NO: 34 (CDR3), and SEQ. ID. NO: 35 (FR4), and SEQ. ID. NO: 36 (FR1), SEQ. ID. NO: 37 (CDR1), SEQ. ID. NO: 38 (FR2), SEQ. ID. NO: 39 (CDR2), SEQ. ID. NO: 40 (FR3), SEQ. ID. NO: 41 (CDR3), and SEQ. ID. NO: 42 (FR4);

for 7G4, SEQ. ID. NO: 43 (FR1), SEQ. ID. NO: 44 (CDR1), SEQ. ID. NO: 45 (FR2), SEQ. ID. NO: 46 (CDR2), SEQ. ID. NO: 47 (FR3), SEQ. ID. NO: 48 (CDR3), and SEQ. ID. NO: 49 (FR4), and SEQ. ID. NO: 50 (FR1), SEQ. ID. NO: 51 (CDR1), SEQ. ID. NO: 52 (FR2), SEQ. ID. NO: 53 (CDR2), SEQ. ID. NO: 54 (FR3), SEQ. ID. NO: 55 (CDR3), and SEQ. ID. NO: 56 (FR4);

for 5H2, SEQ. ID. NO: 57 (FR1), SEQ. ID. NO: 58 (CDR1), SEQ. ID. NO: 59 (FR2), SEQ. ID. NO: 60 (CDR2), SEQ. ID. NO: 61 (FR3), SEQ. ID. NO: 62 (CDR3), and SEQ. ID. NO: 63 (FR4), and SEQ. ID. NO: 64 (FR1), SEQ. ID. NO: 65 (CDR1), SEQ. ID. NO: 66 (FR2), SEQ. ID. NO: 67 (CDR2), SEQ. ID. NO: 68 (FR3), SEQ. ID. NO: 69 (CDR3), and SEQ. ID. NO: 70 (FR4);

and for 5D9, SEQ. ID. NO: 71 (FR1), SEQ. ID. NO: 72 (CDR1), SEQ. ID. NO: 73 (FR2), SEQ. ID. NO: 74 (CDR2), SEQ. ID. NO: 75 (FR3), SEQ. ID. NO: 76 (CDR3), and SEQ. ID. NO: 77 (FR4), and SEQ. ID. NO: 78 (FR1), SEQ. ID. NO: 79 (CDR1), SEQ. ID. NO: 80 (FR2), SEQ. ID. NO: 81 (CDR2), SEQ. ID. NO: 82 (FR3), SEQ. ID. NO: 83 (CDR3), and SEQ. ID. NO: 84 (FR4).

In one set of embodiments, the present invention provides the full-length, humanized monoclonal antibody of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody in isolated form and in pharmaceutical preparations. Similarly, as described herein, the present invention provides isolated nucleic acids, host cells transformed with nucleic acids, and pharmaceutical preparations including isolated nucleic acids, encoding the full-length, humanized monoclonal antibody of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody. Finally, the present invention provides methods, as described more fully herein, employing these antibodies and nucleic acids in the *in vitro* and *in vivo* diagnosis, prevention and therapy of dengue virus disease.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of a full-length antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the

antigen binding sites of a full-length antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986, *supra*; Roitt, 1991, *supra*). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

The complete amino acid sequences of the antigen-binding Fab portion of the 5H2 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 64-70 disclose the amino acid sequence of the Fd fragment of 5H2. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 64 through SEQ. ID. NO: 70, respectively. SEQ. ID. NOs: 57-63 disclose the amino acid sequence of the light chain of 5H2. The amino acid sequences of the light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 57 through SEQ. ID. NO: 63, respectively.

The complete amino acid sequences of the antigen-binding Fab portion of the 5A7 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 8-14 disclose the amino acid sequence of the Fd fragment of 5A7. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 8 through SEQ. ID. NO: 14, respectively. SEQ. ID. NOs: 1-7 disclose the amino acid sequence of the light chain of 5A7. The amino acid sequences of the light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 1 through SEQ. ID. NO: 7, respectively.

The complete amino acid sequences of the antigen-binding Fab portion of the 3C1 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 22-28 disclose the amino acid sequence of the Fd fragment of 3C1. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 22 through SEQ. ID. NO: 28, respectively. SEQ. ID. NOs: 15-21 disclose the amino acid sequence of the light chain of 3C1. The amino acid sequences of the light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 15 through SEQ. ID. NO: 21, respectively.

The complete amino acid sequences of the antigen-binding Fab portion of the 3E4 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 36-42 disclose the amino acid sequence of the Fd fragment of 3E4. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 36 through SEQ. ID. NO: 42, respectively. SEQ. ID. NOs: 29-35 disclose the amino acid sequence of the light chain of 3E4. The amino acid sequences of the light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 29 through SEQ. ID. NO: 35, respectively.

The complete amino acid sequences of the antigen-binding Fab portion of the 7G4 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 50-56 disclose the amino acid sequence of the Fd fragment of 7G4. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 50 through SEQ. ID. NO: 56, respectively. SEQ. ID. NOs: 43-49 disclose the amino acid sequence of the light chain of 7G4. The amino acid sequences of the light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 43 through SEQ. ID. NO: 49, respectively.

The complete amino acid sequences of the antigen-binding Fab portion of the 5D9 monoclonal antibody as well as the relevant FR and CDR regions are disclosed herein. SEQ. ID. NOs: 78-84 disclose the amino acid sequence of the Fd fragment of 5D9. The amino acid sequences of the heavy chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 78 through SEQ. ID. NO: 84, respectively. SEQ. ID. NOs: 71-77 disclose the amino acid sequence of the light chain of 5D9. The amino acid sequences of the

light chain FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 regions are disclosed as SEQ. ID. NO: 71 through SEQ. ID. NO: 77, respectively.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of full-length antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDRI and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR I and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDRI and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDRI and/or CDR2 regions have been replaced by homologous human or non-human sequences. Thus, those skilled in the art may alter the antibody by the construction of CDR grafted or chimeric antibodies or antibody fragments containing all, or part thereof, of the disclosed heavy and light chain V-region CDR aa sequences (Jones, P.T. *et al.* 1986. *Nature* 321:522; Verhoeven, M. *et al.* 1988 *Science* 39:1534; and Tempest, P.R. *et al.* 1991 *Bio/Technology* 9:266), without destroying the specificity of the antibodies for the dengue type 4 virus epitope. Such CDR grafted or chimeric antibodies or antibody fragments can be effective in prevention and treatment of dengue infection in animals (*e.g.* cattle) and man.

In preferred embodiments, the chimeric antibodies of the invention are humanized chimpanzee monoclonal antibodies including at least the heavy chain CDR3 region of the

5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody. As noted above, such chimeric antibodies may be produced in which some or all of the FR regions of the antibody have been replaced by other homologous human FR regions. In addition, the Fc portions may be replaced so as to produce IgA or IgM as well as IgG antibodies bearing some or all of the of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody. Of particular importance is the inclusion of the heavy chain CDR3 region and, to a lesser extent, the other CDRs of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody. Such humanized chimpanzee monoclonal antibodies will have particular utility in that they will not evoke an immune response against the antibody itself.

It is also possible, in accordance with the present invention, to produce chimeric antibodies including non-human sequences. Thus, one may use, for example, murine, ovine, equine, bovine or other mammalian Fc or FR sequences to replace some or all of the Fc or FR regions of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody. Some of the CDRs may be replaced as well. Again, however, it is preferred that at least the heavy chain CDR3 of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody, be included in such chimeric antibodies and, to a lesser extent, it is also preferred that some or all of the other of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody, be included. Such chimeric antibodies bearing non-human immunoglobulin sequences admixed with the CDRs of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody are not preferred for use in humans and are particularly not preferred for extended use because they may evoke an immune response against the non-human sequences. They may, of course, be used for brief periods or in immunosuppressed individuals but, again, humanized chimpanzee monoclonal antibodies are preferred. Because such antibodies may be used for brief periods or in immunosuppressed subjects, chimeric antibodies bearing non-human mammalian Fc and FR sequences but including at least the heavy chain CDR3 of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody are contemplated as alternative embodiments of the present invention.

For inoculation or prophylactic uses, the antibodies of the present invention are preferably full-length antibody molecules including the Fc region. Such full-length antibodies will have longer half-lives than smaller fragment antibodies (*e.g.* Fab) and are more suitable for intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal administration.

In some embodiments, Fab fragments, including chimeric Fab fragments, are preferred.

In addition to Fabs, smaller antibody fragments and epitope-binding peptides having binding specificity for the epitope defined by the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody are also contemplated by the present invention and can also be used to bind the virus. For example, single chain antibodies can be constructed according to the method of U.S. Pat. No. 4,946,778, to Ladner et al. Single chain antibodies comprise the variable regions of the light and heavy chains joined by a flexible linker moiety. Yet smaller is the antibody fragment known as the single domain antibody or Fd, which comprises an isolated VH single domain. Techniques for obtaining a single domain antibody with at least some of the binding specificity of the full-length antibody from which they are derived are known in the art.

It is possible to determine, without undue experimentation, if an altered or chimeric antibody has the same specificity as the antibody of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody of the invention by ascertaining whether the former blocks the latter from binding to dengue type 4 virus. If the monoclonal antibody being tested competes with the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody as shown by a decrease in binding of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody then it is likely that the two monoclonal antibodies bind to the same, or a closely spaced, epitope. Still another way to determine whether a monoclonal antibody has the specificity of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody of the invention is to pre-incubate the antibody of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody with which it is normally reactive, and then add the monoclonal antibody being tested to determine if the

monoclonal antibody being tested is inhibited in its ability to bind dengue type 4 virus. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a functionally equivalent, epitope and specificity as the antibody of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody of the invention. Screening of monoclonal antibodies of the invention also can be carried out utilizing dengue type 4 virus and determining whether the monoclonal antibody neutralizes dengue type 4 virus.

By using the antibodies of the invention, it is now possible to produce anti-idiotypic antibodies which can be used to screen other monoclonal antibodies to identify whether the antibody has the same binding specificity as an antibody of the invention. In addition, such anti-idiotypic antibodies can be used for active immunization (Herlyn, D. *et al.* 1986 *Science* **232**:100). Such anti-idiotypic antibodies can be produced using well-known hybridoma techniques (Kohler, G. and Milstein, C. 1975 *Nature* **256**:495). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the cell line of interest. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, is responsible for the specificity of the antibody.

An anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The immunized animal will recognize and respond to the idiotype determinants of the immunizing antibody and produce an antibody to these idiotype determinants. By using the anti-idiotypic antibodies of the immunized animal, which are specific for the monoclonal antibodies of the invention, it is possible to identify other clones with the same idio type as the antibody of the hybridoma used for immunization. Idiotype identity between monoclonal antibodies of two cell lines demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using anti-idiotypic antibodies, it is possible to identify other hybridomas expressing monoclonal antibodies having the same epitopic specificity.

It is also possible to use the anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region

which is the image of the epitope bound by the first monoclonal antibody. Thus, the anti-idiotypic monoclonal antibody can be used for immunization, since the anti-idiotypic monoclonal antibody binding domain effectively acts as an antigen.

Nucleic Acids Encoding Anti-DENV-4 Antibodies

Given the disclosure herein of the amino acid sequences of the heavy chain Fd and light chain variable domains of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody, one of ordinary skill in the art is now enabled to produce nucleic acids which encode this antibody or which encode the various fragment antibodies or chimeric antibodies described above. It is contemplated that such nucleic acids will be operably joined to other nucleic acids forming a recombinant vector for cloning or for expression of the antibodies of the invention. The present invention includes any recombinant vector containing the coding sequences, or part thereof, whether for prokaryotic or eukaryotic transformation, transfection or gene therapy. Such vectors may be prepared using conventional molecular biology techniques, known to those with skill in the art, and would comprise DNA coding sequences for the immunoglobulin V-regions of the 5H2 antibody, or the 5A7, 3C1, 3E4, 7G4, or 5D9 antibody or other dengue type 4 virus antibody, including framework and CDRs or parts thereof, and a suitable promoter either with (Whittle, N. *et al.* 1987 *Protein Eng.* 1:499 and Burton, D.R. *et al.* 1994 *Science* 266:1024) or without (Marasco, W.A. *et al.* 1993 *Proc. Natl. Acad. Sci. (USA)* 90:7889 and Duan, L. *et al.* 1994 *Proc. Natl. Acad. Sci. (USA)* 91:5075) a signal sequence for export or secretion. Such vectors may be transformed or transfected into prokaryotic (Huse, W.D. *et al.* 1989 *Science* 246:1275; Ward, S. *et al.* 1989 *Nature* 341:544; Marks, J.D. *et al.* 1991 *J. Mol. Biol.* 222:581; and Barbas, C.F. *et al.* 1991 *Proc. Natl. Acad. Sci. (USA)* 88:7987) or eukaryotic (Whittle, N. *et al.* 1987 *Protein Eng.* 1:499 and Burton, D.R. *et al.* 1994 *Science* 266:1024) cells or used for gene therapy (Marasco, W.A. *et al.* 1993 *Proc. Natl. Acad. Sci. (USA)* 90:7889 and Duan, L. *et al.* 1994 *Proc. Natl. Acad. Sci. (USA)* 91:5075) by conventional techniques, known to those with skill in the art.

The expression vectors of the present invention include regulatory sequences operably joined to a nucleotide sequence encoding one of the antibodies of the invention. As used herein, the term "regulatory sequences" means nucleotide sequences which are necessary for

or conducive to the transcription of a nucleotide sequence which encodes a desired polypeptide and/or which are necessary for or conducive to the translation of the resulting transcript into the desired polypeptide. Regulatory sequences include, but are not limited to, 5' sequences such as operators, promoters and ribosome binding sequences, and 3' sequences such as polyadenylation signals. The vectors of the invention may optionally include 5' leader or signal sequences, 5' or 3' sequences encoding fusion products to aid in protein purification, and various markers which aid in the identification or selection of transformants. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art. The subsequent purification of the antibodies may be accomplished by any of a variety of standard means known in the art.

A preferred vector for screening monoclonal antibodies, but not necessarily preferred for the mass production of the antibodies of the invention, is a recombinant DNA molecule containing a nucleotide sequence that codes for and is capable of expressing a fusion polypeptide containing, in the direction of amino- to carboxy-terminus, (1) a prokaryotic secretion signal domain, (2) a polypeptide of the invention, and, optionally, (3) a fusion protein domain. The vector includes DNA regulatory sequences for expressing the fusion polypeptide, preferably prokaryotic, regulatory sequences. Such vectors can be constructed by those with skill in the art and have been described by Smith, G.P. *et al.* (1985 *Science* **228**:1315); Clackson, T. *et al.* (1991 *Nature* **352**:624); Kang *et al.* (1991 in "Methods: A Companion to Methods in Enzymology: Vol. 2"; R.A. Lerner and D.R. Burton, ed. Academic Press, NY, pp 111-118); Barbas, C.F. *et al.* (1991 *Proc. Natl. Acad. Sci. (USA)* **88**:7978), Roberts, B.L. *et al.* (1992 *Proc. Natl. Acad. Sci. (USA)* **89**:2429).

A fusion polypeptide may be useful for purification of the antibodies of the invention. The fusion domain may, for example, include a poly-His tail which allows for purification on Ni²⁺ columns or the maltose binding protein of the commercially available vector pMAL (New England BioLabs, Beverly, MA). A currently preferred, but by no means necessary, fusion domain is a filamentous phage membrane anchor. This domain is particularly useful for screening phage display libraries of monoclonal antibodies but may be of less utility for the mass production of antibodies. The filamentous phage membrane anchor is preferably a domain of the cpIII or cpVIII coat protein capable of associating with the matrix of a

filamentous phage particle, thereby incorporating the fusion polypeptide onto the phage surface, to enable solid phase binding to specific antigens or epitopes and thereby allow enrichment and selection of the specific antibodies or fragments encoded by the phagemid vector.

The secretion signal is a leader peptide domain of a protein that targets the protein to the membrane of the host cell, such as the periplasmic membrane of Gram-negative bacteria. A preferred secretion signal for *E. coli* is a *pelB* secretion signal. The leader sequence of the *pelB* protein has previously been used as a secretion signal for fusion proteins (Better, M. *et al.* 1988 *Science* **240**:1041; Sastry, L. *et al.* 1989 *Proc. Natl. Acad. Sci. (USA)* **86**:5728; and Mullinax, R.L. *et al.*, 1990 *Proc. Natl. Acad. Sci. (USA)* **87**:8095). Amino acid residue sequences for other secretion signal polypeptide domains from *E. coli* useful in this invention can be found in Neidhard, F.C. (ed.), 1987 Escherichia coli and Salmonella Typhimurium: Typhimurium Cellular and Molecular Biology, American Society for Microbiology, Washington, D.C.

To achieve high levels of gene expression in *E. coli*, it is necessary to use not only strong promoters to generate large quantities of mRNA, but also ribosome binding sites to ensure that the mRNA is efficiently translated. In *E. coli*, the ribosome binding site includes an initiation codon (AUG) and a sequence 3-9 nucleotides long located 3-11 nucleotides upstream from the initiation codon. The sequence, which is called the Shine-Dalgarno (SD) sequence, is complementary to the 3' end of *E. coli* 16S rRNA. Binding of the ribosome to mRNA and the sequence at the 3' end of the mRNA can be affected by several factors:

The degree of complementarity between the SD sequence and 3' end of the 16S rRNA;

The spacing lying between the SD sequence and the AUG. Optimization is achieved by measuring the level of expression of genes in plasmids in which this spacing is systematically altered. Comparison of different mRNAs shows that there are statistically preferred sequences from positions -20 to +13 (where the A of the AUG is position 0); and

The nucleotide sequence following the AUG, which affects ribosome binding. The 3' regulatory sequences define at least one termination (stop) codon in frame with and operably joined to the heterologous fusion polypeptide.

In preferred embodiments with a prokaryotic expression host, the vector utilized includes a prokaryotic origin of replication or replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such origins of replication are well known in the art. Preferred origins of replication are those that are efficient in the host organism. A preferred host cell is *E. coli*. For use of a vector in *E. coli*, a preferred origin of replication is ColEI found in pBR322 and a variety of other common plasmids. Also preferred is the p15A origin of replication found on pACYC and its derivatives. The ColEI and p15A replicons have been extensively utilized in molecular biology, are available on a variety of plasmids and are described by Sambrook, et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press.

In addition, those embodiments that include a prokaryotic replicon preferably also include a gene whose expression confers a selective advantage, such as drug resistance, to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin, tetracycline, neomycin/kanamycin or chloramphenicol. Vectors typically also contain convenient restriction sites for insertion of translatable DNA sequences. Exemplary vectors are the plasmids pUC18 and pUC19 and derived vectors such as those commercially available from suppliers such as Invitrogen, (San Diego, CA).

When the antibodies of the invention include both heavy chain and light chain sequences, these sequences may be encoded on separate vectors or, more conveniently, may be expressed by a single vector. The heavy and light chain may, after translation or after secretion, form the heterodimeric structure of natural antibody molecules. Such a heterodimeric antibody may or may not be stabilized by disulfide bonds between the heavy and light chains.

A vector for expression of heterodimeric antibodies, such as the full-length antibodies of the invention or the F(ab')₂, Fab or Fv fragment antibodies of the invention, is a recombinant DNA molecule adapted for receiving and expressing translatable first and second DNA sequences. That is, a DNA expression vector for expressing a heterodimeric antibody provides a system for independently cloning (inserting) the two translatable DNA

sequences into two separate cassettes present in the vector, to form two separate cistrons for expressing the first and second polypeptides of a heterodimeric antibody. The DNA expression vector for expressing two cistrons is referred to as a di-cistronic expression vector.

Preferably, the vector comprises a first cassette that includes upstream and downstream DNA regulatory sequences operably joined via a sequence of nucleotides adapted for directional ligation to an insert DNA. The upstream translatable sequence preferably encodes the secretion signal as described above. The cassette includes DNA regulatory sequences for expressing the first antibody polypeptide that is produced when an insert translatable DNA sequence (insert DNA) is directionally inserted into the cassette via the sequence of nucleotides adapted for directional ligation.

The dicistronic expression vector also contains a second cassette for expressing the second antibody polypeptide. The second cassette includes a second translatable DNA sequence that preferably encodes a secretion signal, as described above, operably joined at its 3' terminus via a sequence of nucleotides adapted for directional ligation to a downstream DNA sequence of the vector that typically defines at least one stop codon in the reading frame of the cassette. The second translatable DNA sequence is operably joined at its 5' terminus to DNA regulatory sequences forming the 5' elements. The second cassette is capable, upon insertion of a translatable DNA sequence (insert DNA), of expressing the second fusion polypeptide comprising a secretion signal with a polypeptide coded by the insert DNA.

The antibodies of the present invention may additionally, of course, be produced by eukaryotic cells such as CHO cells, human or mouse hybridomas, immortalized B-lymphoblastoid cells, and the like. In this case, a vector is constructed in which eukaryotic regulatory sequences are operably joined to the nucleotide sequences encoding the antibody polypeptide or polypeptides. The design and selection of an appropriate eukaryotic vector is within the ability and discretion of one of ordinary skill in the art. The subsequent purification of the antibodies may be accomplished by any of a variety of standard means known in the art.

The antibodies of the present invention may furthermore, of course, be produced in plants. In 1989, Hiatt *et al.*, 1989, *Nature* **342**:76 first demonstrated that functional antibodies

could be produced in transgenic plants. Since then, a considerable amount of effort has been invested in developing plants for antibody (or "plantibody") production (for reviews see Giddings, G. *et al.*, 2000 *Nat Biotechnol* 18:1151; Fischer, R. and Emans, N., 2000, *Transgenic Res* 9:279). Recombinant antibodies can be targeted to seeds, tubers, or fruits, making administration of antibodies in such plant tissues advantageous for immunization programs in developing countries and worldwide.

In another embodiment, the present invention provides host cells, both prokaryotic and eukaryotic, transformed or transfected with, and therefore including, the vectors of the present invention.

Diagnostic and Pharmaceutical Anti-DENV-4 Antibody Preparations

The invention also relates to a method for preparing diagnostic or pharmaceutical compositions comprising the monoclonal antibodies of the invention or polynucleotide, sequences encoding the antibodies of the invention or part thereof, the pharmaceutical compositions being used for immunoprophylaxis or immunotherapy of dengue virus disease. The pharmaceutical preparation includes a pharmaceutically acceptable carrier. Such carriers, as used herein, means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

A preferred embodiment of the invention relates to monoclonal antibodies whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 69, and/or whose light chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 62; whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 13, and/or whose light chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 6; whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 27, and/or whose light chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 20; whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 41, and/or whose light chains comprise in CDR3 the

polypeptide having SEQ. ID. NO: 34; whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 55, and/or whose light chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 48; whose heavy chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 83, and/or whose light chains comprise in CDR3 the polypeptide having SEQ. ID. NO: 76; and conservative variations of these peptides. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies having the substituted polypeptide also bind dengue type 4 virus. Analogously, another preferred embodiment of the invention relates to polynucleotides which encode the above noted heavy chain polypeptides and to polynucleotide sequences which are complementary to these polynucleotide sequences. Complementary polynucleotide sequences include those sequences that hybridize to the polynucleotide sequences of the invention under stringent hybridization conditions.

The anti-dengue type 4 virus antibodies of the invention may be labeled by a variety of means for use in diagnostic and/or pharmaceutical applications. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibodies of the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibodies of the invention can be done using standard techniques common to those of ordinary skill in the art.

Another labeling technique which may result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically altered by means of a second reaction. For example, it is common to use haptens

such as biotin, which reacts with avidin, or dinitrophenol, pyridoxal, or fluorescein, which can react with specific antihapten antibodies.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise a monoclonal antibody of the invention that is, or can be, detectably labeled. The kit may also have containers containing buffer(s) and/or a container comprising a reporter-means, such as a biotin-binding protein, such as avidin or streptavidin, bound to a reporter molecule, such as an enzymatic or fluorescent label.

In vitro Detection and Diagnostics

The monoclonal antibodies of the invention are suited for *in vitro* use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize the monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of dengue type 4 virus. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylase, natural and modified cellulose, polyacrylamide, agarose and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

For purposes of the invention, dengue type 4 virus may be detected by the monoclonal antibodies of the invention when present in biological fluids and tissues. Any sample containing a detectable amount of dengue type 4 virus can be used. A sample can be a liquid such as blood, urine, saliva, cerebrospinal fluid, blood, serum or the like; a solid or semi-solid such as tissues, feces, or the like; or, alternatively, a solid tissue such as those commonly used in histological diagnosis.

In vivo Detection of DENV-4

In using the monoclonal antibodies of the invention for the *in vivo* detection of antigen, the detectably labeled monoclonal antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the dengue type 4 virus antigen for which the monoclonal antibodies are specific.

The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to dengue type 4 virus is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled monoclonal antibody for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.01 mg/kg to about 50 mg/kg, preferably 0.1 mg/kg to about 20 mg/kg, most preferably about 0.1 mg/kg to about 2 mg/kg. Such dosages may vary, for example, depending on whether multiple injections are given, on the tissue being assayed, and other factors known to those of skill in the art.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting an appropriate radioisotope. The radioisotope chosen must have a type of decay which is detectable for the given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough such that it is still detectable at the time of maximum uptake by the target, but short enough such that deleterious radiation with respect to the host is acceptable. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission but produce a large

number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions are the bifunctional chelating agents such as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetra-acetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In , ^{97}Ru , ^{67}Ga , ^{68}Ga , ^{72}As , ^{89}Zr and ^{201}Tl .

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd , ^{55}Mn , ^{162}Dy , ^{52}Cr and ^{56}Fe .

The monoclonal antibodies of the invention can be used *in vitro* and *in vivo* to monitor the course of dengue virus disease therapy. Thus, for example, by measuring the increase or decrease in the number of cells infected with dengue type 4 virus or changes in the concentration of dengue type 4 virus present in the body or in various body fluids, it would be possible to determine whether a particular therapeutic regimen aimed at ameliorating dengue virus disease is effective.

Prophylaxis and Therapy of Dengue Virus Disease

The monoclonal antibodies can also be used in prophylaxis and as therapy for dengue virus disease in humans. The terms, "prophylaxis" and "therapy" as used herein in conjunction with the monoclonal antibodies of the invention denote both prophylactic as well as therapeutic administration and both passive immunization with substantially purified polypeptide products, as well as gene therapy by transfer of polynucleotide sequences encoding the product or part thereof. Thus, the monoclonal antibodies can be administered to high-risk subjects in order to lessen the likelihood and/or severity of dengue virus disease or administered to subjects already evidencing active dengue virus infection. In the present invention, Fab fragments also bind or neutralize dengue type 4 virus and therefore may be

used to treat dengue virus infection but full-length antibody molecules are otherwise preferred.

As used herein, a "prophylactically effective amount" of the monoclonal antibodies of the invention is a dosage large enough to produce the desired effect in the protection of individuals against dengue virus infection for a reasonable period of time, such as one to two months or longer following administration. A prophylactically effective amount may vary from about 0.01 mg/kg to about 50 mg/kg, preferably from about 0.1 mg/kg to about 20 mg/kg, most preferably from about 0.2 mg/kg to about 2 mg/kg, in one administration.

As used herein, a "therapeutically effective amount" of the monoclonal antibodies of the invention is a dosage large enough to produce the desired effect in which the symptoms of dengue virus disease are ameliorated or the likelihood of infection is decreased. A therapeutically effective amount is not, however, a dosage so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, a therapeutically effective amount may vary with the subject's age, condition, and sex, as well as the extent of the disease in the subject and can be determined by one of skill in the art. The dosage may be adjusted by the individual physician or veterinarian in the event of any complication. A therapeutically effective amount may vary from about 0.01 mg/kg to about 50 mg/kg, preferably from about 0.1 mg/kg to about 20 mg/kg, most preferably from about 0.2 mg/kg to about 2 mg/kg, in one or more dose administrations daily, for one or several days. Preferred is administration of the antibody for 2 to 5 or more consecutive days in order to avoid "rebound" of virus replication from occurring.

The monoclonal antibodies of the invention can be administered by injection or by gradual infusion over time. The administration of the monoclonal antibodies of the invention may, for example, be intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. Techniques for preparing injectate or infusate delivery systems containing antibodies are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Remington's Pharmaceutical Sciences, 18th edition, 1990, Mack Publishing). Those of skill in the art can readily

determine the various parameters and conditions for producing antibody injectates or infusates without resort to undue experimentation.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and the like.

Chimpanzee antibody response to intrahepatic transfection with DENV-4 RNA and to subsequent inoculation with a mixture of DENV-1, DENV-2 and DENV-3 viruses.

Two chimpanzees (# 1616 and #1618) were intrahepatically transfected with the full-length RNA transcripts of cloned DENV-4 cDNA (Lai, C. J. *et al.* 1991 *Proc. Natl. Acad. Sci. USA.* **88**:5139-5143). Four weeks after inoculation, these chimpanzees showed transient mild serum ALT elevations and became sero-positive for DENV-4, indicating that both animals were infected. At 9 weeks, the antibodies against DENV-4 reached a PRNT₅₀ titer of 1/ 992 and 1/1065, respectively. This level of neutralizing antibodies was comparable to that in rhesus monkeys infected with DENV-4 by a subcutaneous route (Men R., *et al.* 1996 *J. Virol.* **70**:3930-3937). To increase the repertoire of dengue virus-specific antibodies, both chimpanzees were inoculated with a mixture of DENV-1, DENV-2 and DENV-3 viruses, each at 10⁶ pfu/dose, ten-and-half months after DENV-4 RNA transfection. Both chimpanzees developed moderate to high PRNT₅₀ titers of antibodies against DENV-1, DENV-2 and DENV-3 (Table 1), indicating that the chimpanzees were infected with each of these viruses. Meanwhile, the PRNT₅₀ antibody titer against DENV-4 increased approximately 2 fold following infection with DENV-1, DENV-2 and DENV-3 viruses.

Chimpanzee γ 1/ κ combinatorial Fab antibody libraries.

Two phagemid libraries were constructed from bone marrow mRNA of chimpanzee #1618: (A) Library D4 was prepared from the chimpanzee after intra-hepatic inoculation with DENV-4 RNA; and (B) Library D1-4 was prepared from the same animal after infection with a mixture of the other three dengue serotype viruses. Phage library D4 was panned three successive rounds against DENV-4 virions immobilized directly in an ELISA plate. After the third panning, plasmid was isolated and cleaved with Spe I and Nhe I for expression of soluble Fabs. Library D1-4 was panned three successive rounds against DENV-4 virions captured by chimpanzee antibodies coated on an ELISA plate. In this manner, possible conformational distortions of the DENV-4 virion surface due to direct coating on a solid phase might be minimized. Similarly, after the third panning, plasmid was isolated and cleaved with Spe I and Nhe I for expression of soluble Fabs.

Identification and characterization of chimpanzee Fabs specific to DENV-4.

E. coli transformants were screened for production of soluble Fabs capable of binding to DENV-4. Plasmid containing the Fab insert was analyzed by digestion with BstN I in order to select distinct clones. Sequence analysis of the V_H and V_L DNA inserts identified Fabs 5A7, 3C1, 3E4, and 7G4 in library D4. Fabs 5H2 and 5D9, which varied in the V_L sequences but shared a nearly identical V_H sequence (a single amino acid difference in the FR3 region), were recovered from library D1-4 (Figure 2). The sequences in the heavy chain complementarity-determining region 3 (CDR3) (Wu, T. *et al.* 1993 *Proteins: Structure, Functions and Genetics* 16:1-7), critical for antigen binding, showed a greater diversity than the sequences in other regions among these Fabs. A sequence similarity search of the available human immunoglobulin genes was conducted to determine the specific germ line origin of these chimpanzee Fab fragments. The chimpanzee V_H and V_L sequences and their most related human immunoglobulin genes of the germ line VH or $V\kappa$ families are shown (Table 2). These chimpanzee V_H or V_L sequences and their human homologues shared 88-95% identity excluding the CDR3 region.

Antigenic specificity of chimpanzee Fab monoclonal antibodies.

First, the binding activity of the Fab antibodies to DENV-4 was analyzed by ELISA. All six selected Fabs showed strong binding to DENV-4 virions (Table 3). Chimpanzee Fab

1F2, which was selected from library D4 for its ability to bind anti-human F(ab)² but not DENV-4, was used as the control. A cross-reactivity to DENV-1, DENV-2 or DENV-3 was detected for Fabs 3E4, 7G4 and 5A7. Fab 3C1 also cross-reacted with DENV-2 at a low titer. Fabs 5H2 and 5D9 showed no detectible cross-reactivity to DENV-1, DENV-2 or DENV-3 virus. Radio-immunoprecipitation using a lysate of DENV-4 infected Vero cells was then performed to determine the antigen-binding specificity (Fig. 3A). Fab 5A7 selectively precipitated prM. All other Fabs precipitated both E and prM. The amount of prM relative to E precipitated varied, depending on the Fab. Radio-immunoprecipitation was again performed using labeled E or prM prepared individually in recombinant vaccinia virus-infected cells (Fig. 3B). Fabs 3E4 and 7G4 precipitated E, but not prM. Fab 3C1 precipitated neither E nor prM. Fab 5D9 precipitated E, but not prM, whereas Fab 5H2 precipitated E and a trace of prM. When the labeled antigens were mixed, co-precipitation of prM and E was again detected for Fabs 3E4, 7G4, 3C1 and 5H2.

Mapping Fab antibody binding sites on DENV-4 virions by competition ELISA.

Biotinylated Fabs 3C1, 3E4, 7G4 and 5H2 were each tested for binding to DENV-4 in the presence of an unlabeled, competing Fab. Chimpanzee Fab 1F2, which did not bind DENV-4, was analyzed in parallel. Fab 5D9, which was nearly identical to Fab 5H2, was not tested. The result (Fig. 4, panels A-D) showed that binding of Fab 3C1 to DENV-4 was competed by Fab 5A7, but not by Fab 3E4, 7G4, 5H2 or 1F2. Thus, the binding site on prM for Fab 3C1 overlapped with that for Fab 5A7. Fab 3E4 and Fab 7G4 also competed with each other for binding to DENV-4, indicating that their binding sites on E overlapped. The binding site on E for Fab 5H2 was unique, as binding competition with other Fabs was not observed.

DENV-4 and cross-serotype neutralizing activity of Fab antibodies.

Affinity-purified Fabs were used for PRNT₅₀ determination (Table 4). Similar to the Fab 1F2 control, prM-specific Fab 5A7 or 3C1 did not neutralize DENV-4. Fabs 3E4 and 7G4 exhibited a low DENV-4-neutralizing activity with a PRNT₅₀ titer at 91 ug/ml or greater. Fab 3E4, which was most cross-reactive to DENV-1, DENV-2 or DENV-3, was used in a cross-serotype neutralization assay. The cross-neutralizing activity against DENV-1, DENV-2 or DENV-3 was lower than that detected for DENV-4. Importantly, Fab 5H2 and

Fab 5D9 neutralized DENV-4 efficiently, with a PRNT₅₀ titer of 0.24 and 0.58 ug/ml, respectively.

Humanized chimpanzee full-length IgG1 antibodies produced in CHO cells.

Production of full-length antibodies from the Fab $\gamma 1/\kappa$ sequences was achieved with expression vector pFab CMV-dhfr, which provides a portion of the hinge and the entire C_H2 and C_H3 sequences of the human gamma-1 heavy chain (Fig. 1). A *dhfr* gene was inserted into the vector for selection of antibody-producing CHO cells with methotrexate and for gene copy amplification. Other modifications of the expression vector included conversion of the chimpanzee-specific hinge sequence to the human counterpart and an A to G substitution at the last nucleotide of the intron between CH₂ and CH₃ exons of the heavy chain sequence. Thus, the product was a full-length, chimeric human-chimpanzee (humanized) IgG1 antibody. Fab 5H2 was chosen for conversion to the whole IgG1 antibody. The full-length IgG1 5H2 was secreted into the culture medium of the transformed CHO cells and the yield of the affinity-purified product was approximately 1.8 mg per liter. Affinity-purified IgG1 5H2 was compared with Fab 5H2 for binding affinity to DENV-4 by ELISA. The IgG1 5H2 and Fab 5H2 had equilibrium affinity constants (*K_d*) of 0.24 nM and 0.42 nM, respectively. IgG1 5H2 neutralized three DENV-4 strains from two geographic regions *in vitro* at a similar high PRNT₅₀ titer of 0.03-0.05 ug/ml (Fig. 5). Humanized IgG1 5H2 represents the first DENV-4-neutralizing monoclonal antibody of primate origin.

Discussion.

The last few decades have seen the isolation and characterization of a large number of murine monoclonal antibodies against the four dengue viruses and other arthropod-borne flaviviruses. The clinical utility of these murine monoclonal antibodies is limited, because of their propensity to induce an antibody response in humans. To develop a strategy of passive immunization against dengue, we turned to antibodies from chimpanzees, which are closely related to humans and can be experimentally infected with dengue viruses. The current study represents the first successful recovery of chimpanzee Fab monoclonal antibodies against the dengue virus by combinatorial cloning.

Analysis of the series of Fab antibodies against DENV-4 recovered by combinatorial cloning suggested a pattern of chimpanzee antibody response to intrahepatic infection with

the infectious DENV-4 RNA transcripts. As in dengue virus infection of mice, both PrM-specific (Fab 5A7) and E-specific (Fabs 3E4 and 7G4) antibodies were identified in the chimpanzee. Interestingly, both Fab 3E4 and Fab 7G4 antibodies co-precipitated prM and E, when the two antigens were mixed. Fab 3C1 also co-precipitated prM and E, but did not precipitate either when these antigens were present individually. These results suggest that these Fabs recognized either PrM or E in the prM-E heterodimer. These chimpanzee Fab antibodies are contemplated to be useful for analysis of PrM-E interactions and the antigenic structure of dengue virus. Nevertheless, their DENV-4-neutralizing activity was low or not detected and they are not likely to be effective against the virus.

Our goal of recovering antibodies highly efficient for neutralizing DENV-4 was achieved by repertoire cloning of chimpanzee bone marrow following multiple dengue virus infections. In this case, DENV-4 virions captured by polyclonal antibodies immobilized on plates were used for phage panning. The panning modification might better preserve the native conformation of the DENV-4 antigenic structure. This experiment yielded Fabs 5H2 and 5D9 that neutralized DENV-4 efficiently at a PRNT₅₀ titer in the range of 0.2-0.6 µg/ml. Both Fabs shared a nearly identical V_H sequence, but varied in the CDR1, CDR2 and other regions of their V_L sequences. These differences in the V_L and V_H sequences could explain the observation that Fab 5H2 co-precipitated E and prM, whereas Fab 5D9 precipitated only E. Importantly, both Fabs neutralized DENV-4 efficiently at a PRNT₅₀ titer in the range of that of human Fab antibodies against the respiratory syncytial virus (Crowe, J. E. Jr. *et al.* 1994. *Proc. Natl. Acad. Sci.* 91:1386-1390), Ebola virus (Maruyama, T. *et al.* 1999 *J. Virol.* 73:6024-6030) or human immunodeficiency virus (Barbas, C. F. *et al.* 1994 *Proc. Nat. Acad. Sci.* 91:3809-3813) selected by phage display.

Fab antibody fragments have a rapid clearance rate in humans, and therefore are not preferred for clinical use. Conversion of the Fab fragments to their whole IgG1 antibody molecules was achieved using expression vector pFab CMV-dhfr, which provided a portion of the hinge and the complete C_H2 and C_H3 heavy chain sequences from a human germ-line DNA segment. A dihydrofolate reductase gene was inserted in the expression vector to increase the IgG1 antibody production. The chimpanzee-specific sequence found in the hinge region was also converted to the human sequence. The humanized IgG1 5H2 had at least

equal binding affinity for DENV-4, if not higher than Fab 5H2, as measured by equilibrium affinity constants. Importantly, the humanized antibody IgG1 5H2 exhibited a PRNT₅₀ titer of 0.03-0.05 ug/ml, approximately 8 fold more efficient than that of the Fab fragment against DENV-4.

Polyclonal antibody preparations against Caribbean DENV-4 isolates, including strain 814669, have been shown to neutralize DENV-4 H241 isolated from the Philippines less efficiently than the homologous DENV-4 strains, suggesting that there are antigenic variations among DENV-4 strains with different geographical origins (Henchal, E. A. *et al.* 1986 *Am. J. Trop. Med. Hyg.* 35:393-400). Sequence analysis also indicates that there is a significant genetic variation among DENV-4 isolates from different geographic regions (Lanciotti, R. S. *et al.* 1997 *J. Gen. Virol.* 78:2279-2284). Thus, it is significant that IgG1 5H2 was able to neutralize geographically diverse DENV-4 isolates at a similar high titer. The DENV-4-neutralizing activity of IgG1 5H2 was approximately 6-10 fold higher than the IgG monoclonal antibody against the Ebola virus (Maruyama, T. *et al.* 1999 *J. Virol.* 73:6024-6030) and 40-60 fold higher than the humanized mouse antibody (MEDI-493) against respiratory syncytial virus (Johnson, S. *et al.* 1997 *J. Inf. Dis.* 176:1215-1224). The CHO cell line obtained in this study produced the humanized chimpanzee antibody IgG1 5H2 at approximately 1.8 mg per liter. Increased production of this antibody in other mammalian cell systems is anticipated.

A computer search revealed that the amino acid sequences of V_H and V_L segments of these Fab antibodies shared a strong homology, ranging from 88-95 %, with the sequences of their human immunoglobulin homologues. In particular, the Fab 5H2 γ 1 heavy chain and κ light chain sequences shared 89% and 94% sequence similarity to the human germ line IgG gene homologues, excluding the CD3 region (Pech, M. *et al.* 1985 *J. Mol. Biol.* 183:291-299; Tomlinson, I.M. *et al.* 1992 *J. Mol. Biol.* 227:776-798). Furthermore, in the C_H1 or C_L region there was only one amino acid (or approximately 1.0 %) difference between chimpanzee and human sequences (Ehrlich, P. H., *et al.* 1990 *Hum. Antibody Hybridoma* 1:23-26; Takahashi, N., S. *et al.* 1982 *Cell* 29:671-679). The high level of antibody sequence similarity and a number of other observations addressing this issue predict that chimpanzee antibodies may be administered directly to humans without further modifications to humanize these reagents

(Ehrlich, P. H. *et al.* 1990 *Hum. Antibody Hybridoma* 1:23-26; Ehrlich, P. H. *et al.* 1990 *Hybridoma* 7:385-395). Experimental data available indicate that little immunogenicity is seen when components of human antibodies are introduced into chimpanzees (Ogata, N. *et al.* 1993 *Proc. Natl. Acad. Sci.* 90:3014-3018).

The cause of severe dengue sometimes associated with secondary dengue virus infection remains controversial. According to one hypothesis, in a secondary infection dengue virus could form a complex with a sub-neutralizing level of cross-reactive antibodies produced during the primary infection, leading to an enhanced uptake and replication in susceptible mononuclear cells via their Fc receptors (Halstead, S. 1982 *Progress in Allergy*. 31:301-364). Several classes of FcR receptors have been identified on the cell surface and their interacting amino acids in the respective IgG have been carefully mapped (Allen, J. M., and B. Seed 1989 *Science* 243:378-381; Chappel, S. M. *et al.* 1991. *Proc. Natl. Acad. Sci.* 88:9036-9040). It is now possible to ablate the FcR receptor binding sequences in the antibody molecules and to test their activity for enhancing dengue virus replication *in vitro* (Armour, K. L. *et al.* 1999 *Eur. J. Immunol.* 29:2613-2624; Schlesinger, J. J. and S. Chapman 1999 *Virology* 260:84-88; Shields, R. L. *et al.* 2001 *J. Biol. Chem.* 276:6591-6604). Humanized chimpanzee IgG1 monoclonal antibodies lacking the FcR1 binding sequences should permit a critical test of the hypothesis. For clinical application, it is important that these humanized neutralizing antibodies not enhance dengue virus replication in human monocytes or other FcR receptor-bearing cells. As specific treatment for severe dengue is still not available, an early intervention during the viremic phase by passive transfer with highly efficient neutralizing antibodies is anticipated to prove beneficial.

Since there are four dengue virus serotypes, monoclonal antibodies against each of the remaining three dengue serotypes will be required for effective prevention of dengue infection using this approach. It should be possible similarly to identify Fab antibodies from the infected chimpanzees that efficiently neutralize each of the other three dengue virus serotypes. Accordingly, a panel of humanized chimpanzee monoclonal antibodies that most efficiently neutralize these dengue viruses will be prepared and evaluated for protection against dengue infection in animal models and ultimately in humans such that the

monoclonal antibody against dengue type 4 virus is predicted to be invaluable as an intermediate and a final tetravalent vaccine.

Table 1. Serum neutralizing antibody titers of chimpanzees that were previously inoculated with DENV-4 RNA intra-hepatically and then infected with a mixture of DENV-1, DENV-2 and DENV-3 viruses nine months later.

| Chimpanzee e | Infection with DENV-1-3 | <u>Neutralizing antibody titer against</u> | | | |
|-----------------|----------------------------|--|--------|--------|--------|
| | | DENV-4 | DENV-1 | DENV-2 | DENV-3 |
| 1616 | Pre- | 1031 | <10 | 34 | 80 |
| | Post- | 2380 | 327 | 880 | 610 |
| 1618 | Pre- | 1110 | 23 | 69 | 156 |
| | Post- | 1654 | 730 | 1787 | 1271 |

Chimpanzees were infected with a mixture of DENV-1, DENV-2 and DENV-3 at a dose of 10^6 pfu, each virus. The neutralizing antibody titer was the reciprocal of serum dilution that yielded 50% plaque reduction.

Table 2. Sequence similarities between chimpanzee Fab antibodies and their most related human germ line immunoglobulin genes.

| Chimp. Fab | V _H Homologue | | Ref. cited | V _L Homologue | | Ref. Cited |
|---------------|--------------------------|------------|---------------|--------------------------|------------|---------------|
| | Family (gene) | % Identity | | Family (gene) | % Identity | |
| 5A7 | VH3 (COS-6) | 95 | <i>a</i> | Vκ3 (DPK-23) | 90 | <i>B</i> |
| 3C1 | VH1 (DP-10) | 88 | <i>a</i> | Vκ1 (L12a) | 92 | <i>C</i> |
| 3E4 | VH1 (DP-10) | 88 | <i>a</i> | Vκ2 (DPK-8) | 88 | <i>B</i> |
| 7G4 | VH3 (DP-54) | 92 | <i>a</i> | Vκ1 (L12a) | 95 | <i>C</i> |
| 5H2 | VH4 (DP-71) | 89 | <i>a</i> | Vκ1 (Va) | 94 | <i>D</i> |
| 5D9 | VH4 (DP-71) | 88 | <i>a</i> | Vκ1 (Va) | 93 | <i>D</i> |

The DNAPlot program was used to search for the most homologous sequence of human IgG molecules in the data base. % identity in the V_H or V_L region excluding CDR3 is included.

a. Tomlinson, I.M. *et al.* 1992 *J. Mol. Biol.* **227**:776-798

b. Cox, J.P. *et al.* 1994 *Eur. J. Immunol.* **24**:827-836

c. Huber, C. *et al.* 1993 *Eur. J. Immunol.* **23**:2868-2875

d. Pech, M. *et al.* 1985 *J. Mol. Biol.* **183**:291-299

Table 3. Binding activities of Fab monoclonal antibodies to DENV-4 and other dengue virus serotypes as determined by ELISA.

| Fab | ELISA titer of Fab binding to | | | |
|------|-------------------------------|--------|--------|--------|
| | DENV-4 | DENV-1 | DENV-2 | DENV-3 |
| 5A7 | 3.41 | 2.51 | 2.51 | 2.51 |
| 3C1 | 3.71 | 1.30 | 3.11 | 1.30 |
| 3E4 | 4.61 | 4.61 | 4.31 | 4.61 |
| 7G4 | 4.01 | 4.01 | 4.01 | 4.01 |
| 5D9 | 3.41 | <1.0 | <1.0 | <1.0 |
| 5H2 | 4.01 | <1.0 | <1.0 | <1.0 |
| 1F2* | 1.30 | <1.0 | <1.0 | <1.0 |

Microtiter plates were coated with DENV-1, DENV-2, DENV-3 or DENV-4 virions. The starting amount of each Fab in ELISA was approximately 300 µg/ml.

Data are presented as log₁₀ of the reciprocal dilution that gave OD reading 2 fold or higher than the background.

* Chimpanzee Fab from library D4 was used as negative control for binding to DENV-4 and other dengue virus serotypes.

Table 4. DENV-4 neutralizing titer of chimpanzee Fab antibodies

| Fab | Phase library | PRNT ₅₀ titer (μg/ml) |
|------|---------------|----------------------------------|
| 5A7 | D4 | >200 |
| 3C1 | D4 | >200 |
| 7G4 | D4 | 121 |
| 3E4 | D4 | 91 |
| 5D9 | D1-4 | 0.58 |
| 5H2 | D1-4 | 0.24 |
| 1F2* | D4 | >200 |

— Affinity purified chimpanzee Fabs were tested for DENV-4 neutralization by PRNT and the PRNT₅₀ titer was calculated.

* Indicates chimpanzee Fab that did not bind to DENV-4.

Example 1

Preparation of dengue serotype 1-4 viruses.

Mosquito C6/36 cells were grown in MEM supplemented with 10% fetal calf serum. Confluent C6/36 cells were infected with DENV-4 at 0.1 moi in MEM containing 2% fetal calf serum and incubated at 28 C. The medium from the infected cells was harvested at 7 days and again at 10 days. It was clarified by centrifugation at 3,000 rpm in a JA10 rotor (1,000 g) and then centrifuged at 9,000 rpm in a JA10 rotor (15,000 g) overnight. The DENV-4 pellet was re-suspended in phosphate buffered saline (PBS) for phage panning and for ELISA. In addition, DENV-4 grown in C6/36 cells in serum-free medium (VP-SFM, Gibco) was directly used for panning and for ELISA. DENV-1 (Western Pacific strain), DENV-2 (prototype New Guinea C strain) and DENV-3 (strain H87) were prepared in serum-free medium from infected simian Vero cells.

Inoculation of chimpanzees with infectious DENV-4 RNA and with dengue serotype 1, 2, and 3 viruses.

Two dengue virus sero-negative chimpanzees, # 1616 and # 1618, were intrahepatically inoculated with infectious RNA transcripts made from the full-length cDNA clone of DENV-4 strain 814669 (Lai, C. J. *et al.* 1991 *Proc. Natl. Acad. Sci. USA.* **88**:5139-5143). A blood sample was collected weekly from each animal for analysis of the serum ALT levels and for analysis of antibodies to DENV-4. Eleven weeks after DENV-4 RNA inoculation, bone marrow was aspirated from the iliac crest of each chimpanzee and a combinatorial antibody library (designated library D4) was constructed. Eight-and-half months after inoculation with DENV-4 RNA, each of the chimpanzees was inoculated subcutaneously with a mixture of DENV-1, DENV-2 and DENV-3, each at 10^6 plaque forming units (pfu), in 1 ml of minimum essential medium (MEM) (Gibco) plus 0.25% human serum albumin. Six weeks after inoculation with the dengue virus mixture, serum samples were collected for analysis of antibody response. Twelve weeks after inoculation with DENV-1, DENV-2 and DENV-3, bone marrow was aspirated again and a second antibody library (designated library D1-4) was constructed. Both libraries were prepared from bone marrow of chimpanzee #1618, which developed slightly higher antibody titers against DENV-1, DENV-2 and DENV-3 than did chimpanzee #1616.

Construction of $\gamma 1/\kappa$ chimpanzee Fab antibody libraries.

The lymphocytes from bone marrow were separated on a Ficoll-Paque gradient by centrifugation and aliquots of approximately 1×10^7 cells/ml in MEM containing 10% DMSO and 10% fetal calf serum were stored over liquid nitrogen. Total RNA was extracted from 3×10^7 lymphocytes using the RNA Extraction Kit (Stratagene) and mRNA was reverse-transcribed using oligo (dT) as primer (ThermoScript RT-PCR System, Invitrogen). The κ light chain DNA was amplified from the cDNA product by PCR using seven pairs of human κ light chain family-specific 5' primers and a 3' primer in the constant domain (Barbas, C. F. *et al.* 1991 *Proc. Natl. Acad. Sci.* **88**:7978-7982; Glamann, J. *et al.* 1998 *J. Virol.* **72**:585-592; Persson, M. A. *et al.* 1991 *Proc. Natl. Acad. Sci.* **88**:2432-2436; Schofield, D. J. *et al.* 2000 *J. Virol.* **74**:5548-5555). The $\gamma 1$ heavy chain Fd cDNA was amplified using nine human $\gamma 1$ heavy chain family-specific 5' primers plus a chimpanzee $\gamma 1$ -specific 3' primer (Glamann, J. *et al.* 1998 *J. Virol.* **72**:585-592; Schofield, D. J. *et al.* 2000 *J. Virol.* **74**:5548-5555). A thirty-cycle PCR at 94 C for 15 s, 52 C for 50 s and 68 C for 90 s was performed with AmpliTaq DNA polymerase (Perkin Elmers).

Cloning of the chimpanzee κ light chain and $\gamma 1$ heavy chain DNA fragments into the pComb 3H phage display vector was performed as described (Barbas, C. F. *et al.* 1991 *Proc. Natl. Acad. Sci.* **88**:7978-7982; Williamson, R. A. I *et al.* 1993 *Proc. Nat. Acad. Sci.* **90**:41413-4145 [Erratum **91**:1193, 1994]). Briefly, amplified κ light chain DNA fragments were pooled, digested with Sac I and Xba I, and then cloned into pComb 3H (Persson, M. A. *et al.* 1991 *Proc. Natl. Acad. Sci.* **88**:2432-2436) by transformation of electro-competent *E. coli* XL-1 Blue (Stratagene). Plasmid containing the $\gamma 1$ light chain DNA inserts was prepared from *E. coli* transformants and then cleaved with Spe I and Xho I for insertion with amplified $\gamma 1$ heavy chain DNA fragments cleaved with the same enzymes. The plasmid containing both the heavy chain and the light chain DNA inserts was used for transformation of *E. coli* XL-1 Blue by electroporation. In both electroporation steps, the ligated DNA mixture yielded a library size of $1-3 \times 10^8$ *E. coli* colonies.

Panning of phage library and isolation of DENV-4-specific soluble Fabs.

Construction of phage display libraries, recovery and transfer of Fab sequences, and identification of *E. coli* transformants expressing DENV-4-specific soluble Fabs were carried

out as described (Glamann, J. *et al.* 1998 *J. Virol.* 72:585-592; Schofield, D. J. *et al.* 2000 *J. Virol.* 74:5548-5555). Briefly, approximately 10^8 transformants were grown in 2YT broth containing 1% glucose, 10 $\mu\text{g/ml}$ tetracycline and 100 $\mu\text{g/ml}$ ampicillin for 3 hr at 37C. The bacterial culture was then infected with helper phage VSC M13 (Stratagene) at 50 moi to generate the phage library. The phage library D4 was panned by affinity binding on DENV-4 virions coated directly on an ELISA plate that was blocked with 3% nonfat powdered milk in PBS to reduce non-specific binding. The phage library D1-4 was panned by affinity binding on DENV-4 virions captured by a chimpanzee serum immobilized on an ELISA plate to minimize conformational changes of the DENV-4 antigenic structure. Following three cycles of panning, the selected phage mixture was used to infect *E. coli* XL-1 Blue and replicative form DNA (phagemid) was prepared. Phagemid was cleaved with Nhe I and Spe I, and re-circularized to remove the phage gene III portion of the fused Fab sequence. *E. coli* XL-1 Blue were transformed with the circularized DNA and colonies that yielded soluble Fab fragments reactive to DENV-4 virus were screened by ELISA.

DNA sequencing of DENV-4 specific Fab clones.

Plasmid from the selected *E. coli* transformants was initially analyzed by BstNI digestion to identify Fab clones with distinct patterns. Sequence analysis of the Fab V_H and V_L DNA segments was performed on an automated DNA sequencer with the Fluorescence Dideoxynucleotide Terminator Cycle Sequencing Kit using Taq DNA polymerase (Perkin-Elmer). The following primers were used: 5' ACAGCTATCGCGATTGCAGTG (LC-1) and 5' CACCTGATCCTCAGATGGCGG (LC-4) for sequencing the V_L segment; 5' ATTCCTACGGCAGCCGCTGG (HC-1) and 5' GGAAGTAGTCCTTGACCAGGC (HC-4) for sequencing both DNA strands of the V_H segment (Glamann, J. *et al.* 1998 *J. Virol.* 72:585-592; Schofield, D. J. *et al.* 2000 *J. Virol.* 74:5548-5555). Software Vector NTI (InforMax) was used for sequence analysis. The DNAPLOT software program (MRC Center for Protein Engineering) was used to search for human immunoglobulin homologues in the data base.

Production and purification of Fab antibodies.

Selected *E. coli* colonies were grown in 1 liter of L-broth containing 1% glucose and 100 $\mu\text{g/ml}$ ampicillin and 10 $\mu\text{g/ml}$ tetracycline to an early log phase (optical density at 600

nm approximately 0.2) at 30 °C. The bacteria were then transferred to 2 liters of L-broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline and grown at 30°C in the presence of 0.1 mM of inducer IPTG for 5 h. The bacteria were pelleted and resuspended in 20 ml of Extraction Buffer containing 50 mM sodium phosphate, 10 mM Tris-HCl, pH 8.0, 100 mM NaCl (Clontech), and 0.1 mM protease inhibitor AEBSF. After three cycles of freezing and thawing to release the soluble Fab product from the bacterial periplasm, the preparation was clarified by centrifugation at 10,000 rpm in a JA-20 rotor (10,000 g) for 60 min. The histidine-tagged Fab in the supernatant was purified through a column containing 1-ml bed volume of TALON Metal Affinity Resin (Clontech) using the pH elution procedure as suggested by the manufacturer. The Fab purity was verified by polyacrylamide gel electrophoresis using purified human IgG F(ab')₂ (Cappel) as a marker. The Fab concentration was determined colorimetrically using the BCA Protein Assay Kit (Pierce).

Biotinylation of purified Fab fragments and competition ELISA.

Purified Fabs were biotinylated with EZ-Link NHS-LC-Biotin (Pierce) according to the procedure suggested by the supplier. After extensive dialysis against PBS, the biotin-labeled Fab was tested for binding to DENV-4 coated on wells of a microtiter plate. For competition ELISA, a fixed concentration of biotinylated Fab was mixed with a competing Fab in serial dilution and the mixture was added to the DENV-4-coated wells. Streptavidin-alkaline phosphatase was used for detection of biotinylated Fab bound to DEN4.

Radio-labeling of DENV-4 antigens and radio-immunoprecipitation

Infection with DENV-4 or recombinant vaccinia virus and subsequent radio-labeling of infected cells were performed as described earlier (Falgout, B. *et al.* 1990. *J. Virol.* 64:4356-4363). Confluent Vero cells in a T-25 flask were infected with DENV-4 strain 814669 at 1 moi and incubated for 4 days at 37 °C. Infected cells were rinsed once, starved for methionine in methionine-free MEM for 30 min and then labeled with ³⁵S-methionine at 150 µCi/ml (specific activity, 3000 Ci/mM). After a 6-h labeling period, cells were rinsed with cold PBS and lysed in 2 ml radio-immunoprecipitation assay (RIPA) buffer, containing 1% sodium deoxycholate, 1% NP40, 0.1% sodium dodecyl sulphate (SDS), 0.15 M NaCl, 0.1 M Tris, pH 7.5. Confluent CV-1 cells were infected with 5 moi of recombinant vaccinia virus vDENV-4 PrM (Bray, M., and C. J. Lai. 1991 *Virology* 185:505-508) or vDENV-4 E (Men,

R. *et al.* 1991 *J. Virol.* 65:1400-1407) containing the full-length PrM or E coding sequence, respectively, for 15 h at 37 °C. Infected cells were rinsed and starved for methionine in methionine-free MEM, placed in the labeling medium for 2 h, and then lysed in RIPA buffer as described. A 20-μl labeled lysate of DENV-4- or recombinant vaccinia virus-infected cells was mixed with 10 μl of the Fab fragment to be tested and 70 μl RIPA buffer, incubated at 4 °C overnight and then mixed with 2 μl of goat anti-human IgG F(ab')₂ antibody for 2 h. A 100-μl suspension of protein A-Sepharose beads was added to bind the radio-immune complexes. The Sepharose beads were collected by centrifugation and washed three times with RIPA buffer prior to separation by SDS-12% polyacrylamide gel (acrylamide/bisacrylamide ratio of 37.5:1) electrophoresis. Radio-labeled protein bands on the dried gel were visualized by exposure to an X-ray film.

Construction of DNA recombinants and expression of full-length IgG1 in Chinese Hamster Ovary (CHO) cells.

The expression vector pFab CMV (Sanna, P. P. *et al.* 1999 *Immunotechnology* 4:185-188) was re-engineered for IgG1 production (Fig. 1). The vector contained a neomycin phosphotransferase gene (*neo*) located between the two hCMV promoters and a β-lactamase gene (*amp*) between the two poly A sites as mapped by restriction digestion and by sequencing. The *neo* and *amp* locations differed from the published map. A di-hydrofolate reductase (*dhfr*) gene together with the transcription signals was inserted at the unique Not I site in the original vector as the selecting marker and for gene amplification (Wood, C. R. *et al.* 1990 *J. Immunol.* 145:3011-3016). The *dhfr* gene insert was the 1.4 kb DNA fragment from Pvu II/Afe I cleavage of pCDHC68B (Ames, R. S. *et al.* 1995 *J. Immunol. Methods.* 184:177-180). The original plasmid vector contained an A at the last nucleotide position of the intron that precedes the C_H3 exon. This variant nucleotide was converted to G to allow proper RNA splicing for full-length IgG1 expression. The Fab 5H2 κ light chain DNA segment cleaved by Sac I and Xba I was first inserted into the expression vector. The resulting recombinant was then added with the γ1 heavy chain DNA segment cleaved by Xho I and Spe I, which was regenerated by PCR using the Fab 5H2 DNA template and appropriate primers. The chimpanzee-specific sequence in the hinge region together with the variant sequences introduced during plasmid construction were converted to the human hinge

sequence using positive strand primer 5' GACAAACTCACACATGTCCACCGTGCCCA, which introduced a Pci I site (underlined) with silent mutations (Ehrich, P. H. *et al.* 1991 *Mol. Immunol.* **28**:319-322; Takahashi, N. *et al.* 1982 *Cell* **29**:671-679). Accordingly, the IgG1 antibody product would contain the chimpanzee V_H and C_H1 sequences and the entire human hinge, C_H2 and C_H3 sequences.

CHO/dhfr- (duk-) cells were purchased from American Type Culture Collection. Production of the whole IgG1 in CHO/dhfr- cells was carried out by transfection with RsrII-cleaved recombinant plasmid in the presence of Lipofectamine (Gibco). Two days after transfection, cells in a T25 flask were re-plated in Iscove's Modified Dulbecco medium (Gibco) supplemented with 10 % fetal bovine serum plus 10⁻⁷ M methotrexate in the absence of hypoxanthine/thymidine as selecting medium (Dorai, H. and GP Moore. 1987. *J. Immunol.* **139**:4232-4241; Wood, C. R. *et al.* 1990 *J. Immunol.* **145**:3011-3016). Transformed CHO cells resistant to 10⁻⁷ M methotrexate appeared approximately two weeks after transfection. Transformed CHO cells producing IgG1 in the medium were identified by ELISA and by PRNT following sub-cloning in a 96- or 24-well plate. Gene amplification was carried out step-wise by increasing methotrexate concentration to 2x10⁻⁷ M in the selecting medium. CHO cells that produced IgG1 at a high level were selected. The selected CHO cells were adapted to growth in suspension for IgG1 production in serum-free CD CHO medium (Gibco). Medium fluid was concentrated and the IgG1 product was purified through a protein-A affinity column. The full-length IgG1 5H2 antibody was compared with the Fab 5H2 fragment for DENV-4-binding affinity by ELISA. The equilibrium affinity constant (*K_d*) was calculated as the antibody concentration that gave 50% of maximum binding (Lin, C.-W. and S.-C. Wu. 2003 *J. Virol.* **77**:2600-2606; Raffai, R. *et al.* *J. Biol. Chem.* **275**:7109-7116). Determination of DENV-4 neutralizing activity of Fab and whole IgG1 antibodies.

Affinity-purified Fab or full-length IgG1 antibodies were analyzed for DENV-4 neutralizing activity by a modification of plaque reduction neutralization test (PRNT), as described (Okuno, Y. *et al.* 1985 *Arch. Virol.* **86**:129-135). Briefly, approximately 50 focus-forming units of DV-4 were mixed with a serial dilution of Fab or IgG1 antibodies in 250 ul of MEM. The mixture was incubated at 37 C for 30 min and then used for infection of Vero cell monolayers in a 24-well plate. The cells were overlaid with a semi-solid medium

containing 1% Tragacanth gum (Sigma) and incubated at 37 C for 4 days. Foci of DENV-4 infected cells were visualized following immuno-staining with hyperimmune mouse ascites fluid (HMAF) and anti-mouse horseradish peroxidase conjugate (Pierce). The Fab or IgG1 concentration that produced 50% focus reduction was calculated. The neutralizing activity of the IgG1 antibody was tested against DENV-4 strain H241 isolated from the Philippines and two Caribbean DENV-4 isolates, ie, strain 814669 and strain 341750.

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention. All figures, tables, and appendices, as well as patents, applications, and publications, referred to above, are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A substantially pure polypeptide comprising
A chimpanzee Fab antibody fragment obtained by repertoire cloing or its derived humanized monoclonal antibody that binds or neutralizes denge type 4 virus.
2. The substantially pure polypeptide of Claim 1 wherein said antibody fragment comprises an Fd fragment.
3. The substantially pure polypeptide of Claim 1 wherein said antibody fragment comprises an Fab fragment.
4. The substantially pure polypeptide of Claim 1 wherein said antibody includes a heavy chain CDR3 region having the amino acid sequence of SEQ. ID. NO: 69, 55, 41, 27, 13 or 83.
5. The substantially pure polypeptide of Claim 4 wherein said antibody includes a heavy chain CDR2 region having the amino acid sequence of SEQ. ID. NO: 67 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 53 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 39 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 25 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 11 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 81 (when heavy chain CDR3 region is SEQ. ID. NO: 83).
6. The substantially pure polypeptide of Claim 4 wherein said antibody includes a heavy chain CDR1 region having the amino acid sequence of SEQ. ID. NO: 65 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 51 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 37 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 23 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 9 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 79 (when heavy chain CDR3 region is SEQ. ID. NO: 83).
7. The substantially pure polypeptide of Claim 4 wherein said antibody includes a heavy chain Fd region having the amino acid sequence of SEQ. ID. NO: 64-70 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 50-56 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 36-42 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 22-28 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 8-14 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 78-84 (when heavy chain CDR3 region is SEQ. ID. NO: 83).

8. The substantially pure polypeptide of Claim 4 wherein said antibody includes a light chain CDR3 region having the amino acid sequence of SEQ. ID. NO: 62 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 48 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 34 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 20 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 6 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 76 (when heavy chain CDR3 region is SEQ. ID. NO: 83).

9. The substantially pure polypeptide of Claim 4 wherein said antibody includes a light chain CDR2 region having the amino acid sequence of SEQ. ID. NO: 60 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 46 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 32 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 18 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 4 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 74 (when heavy chain CDR3 region is SEQ. ID. NO: 83).

10. The substantially pure polypeptide of Claim 4 wherein said antibody includes a light CDR1 region having the amino acid sequence of SEQ. ID. NO: 58 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 44 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 30 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 16 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 2 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 72 (when heavy chain CDR3 region is SEQ. ID. NO: 83).

11. The substantially pure polypeptide of Claim 4 wherein said antibody includes a light chain region having the amino acid sequence of SEQ. ID. NO: 57-63 (when heavy chain CDR3 region is SEQ. ID. NO: 69), 43-49 (when heavy chain CDR3 region is SEQ. ID. NO: 55), 29-35 (when heavy chain CDR3 region is SEQ. ID. NO: 41), 15-21 (when heavy chain CDR3 region is SEQ. ID. NO: 27), 1-7 (when heavy chain CDR3 region is SEQ. ID. NO: 13), or 71-77 (when heavy chain CDR3 region is SEQ. ID. NO: 83).

12. A substantially pure polypeptide comprising the amino acid sequence of SEQ. ID. NO: 69, 55, 41, 27, 13 or 83.

13. The substantially pure polypeptide of Claim 12 wherein said polypeptide consists essentially of SEQ. ID. NO: 69, 55, 41, 27, 13 or 83.

14. An isolated nucleic acid comprising

A nucleotide sequence encoding a polypeptide selected from the group consisting of the polypeptide of Claim 1, the polypeptide of Claim 2, the polypeptide of Claim 3, the polypeptide of Claim 4, the polypeptide of Claim 5, the polypeptide of Claim 6, the polypeptide of Claim 7, the polypeptide of Claim 8, the polypeptide of Claim 9, the polypeptide of Claim 10, the polypeptide of Claim 11, the polypeptide of Claim 12, and the polypeptide of Claim 13.

15. An isolated nucleic acid as in Claim 14 wherein

Said nucleic acid comprises a vector including a regulatory sequence operably joined to said nucleic acid.

16. A host cell including a vector comprising a nucleic acid of Claim 14.

17. A pharmaceutical preparation comprising

A pharmaceutically acceptable carrier; and

A substantially pure polypeptide selected from the group consisting of the polypeptide of Claim 1, the polypeptide of Claim 2, the polypeptide of Claim 3, the polypeptide of Claim 4, the polypeptide of Claim 5, the polypeptide of Claim 6, the polypeptide of Claim 7, the polypeptide of Claim 8, the polypeptide of Claim 9, the polypeptide of Claim 10, the polypeptide of Claim 11, the polypeptide of Claim 12, and the polypeptide of Claim 13.

18. A diagnostic preparation comprising

A substantially pure polypeptide selected from the group consisting of the polypeptide of Claim 1, the polypeptide of Claim 2, the polypeptide of Claim 3, the polypeptide of Claim 4, the polypeptide of Claim 5, the polypeptide of Claim 6, the polypeptide of Claim 7, the polypeptide of Claim 8, the polypeptide of Claim 9, the polypeptide of Claim 10, the polypeptide of Claim 11, the polypeptide of Claim 12, and the polypeptide of Claim 13;

within a kit.

19. A method for the treatment of dengue virus disease comprising

Administering to a patient a therapeutically effective amount of the pharmaceutical preparation of Claim 17.

20. A method for prophylaxis against dengue virus disease comprising

Administering to a patient a prophylactically effective amount of the pharmaceutical preparation of Claim 17.

21. A method for the diagnosis of dengue virus disease comprising

Administering to a patient an effective amount of the diagnostic preparation of Claim 18, and

Detecting binding of the substantially pure polypeptide as a determination of the presence of dengue virus disease.

22. A method of detecting the presence of dengue virus in a biological sample comprising

Contacting said sample with the diagnostic preparation of Claim 18, and

Assaying binding of the substantially pure polypeptide as a determination of the presence of said dengue virus.

23. Humanized IgG1 5H2 plasmid deposited with ATCC as PTA-5662.

24. pFab CMV-dhfr vector for expression of any full-length IgG1 deposited with ATCC as PTA-5662.

**IDENTIFICATION OF CHIMPANZEE FAB FRAGMENTS BY REPERTOIRE CLONING
AND PRODUCTION OF A FULL-LENGTH HUMANIZED IGG1 ANTIBODY HIGHLY
EFFICIENT FOR NEUTRALIZATION OF DENGUE TYPE 4 VIRUS**

Abstract of the Disclosure

The present invention relates to chimpanzee Fab antibody fragments obtained by repertoire cloning and their derived humanized monoclonal antibodies that bind or neutralize dengue type 4 virus. The invention provides such antibody fragments, such humanized or chimeric antibodies, and pharmaceutical compositions including such antibodies. The invention further provides for isolated nucleic acids encoding the antibodies of the invention and host cells transformed therewith. Additionally, the invention provides for prophylactic, therapeutic, and diagnostic methods employing the antibodies and nucleic acids of the invention.

PATENT

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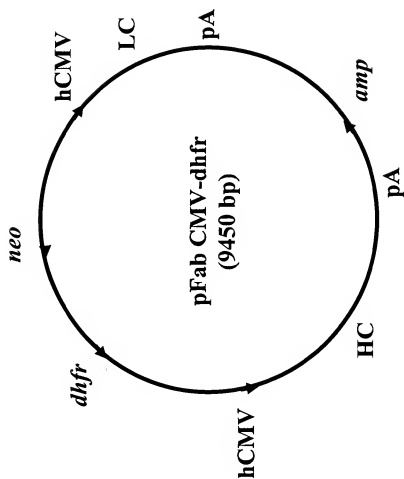


Fig. 1A

Fig. 1B

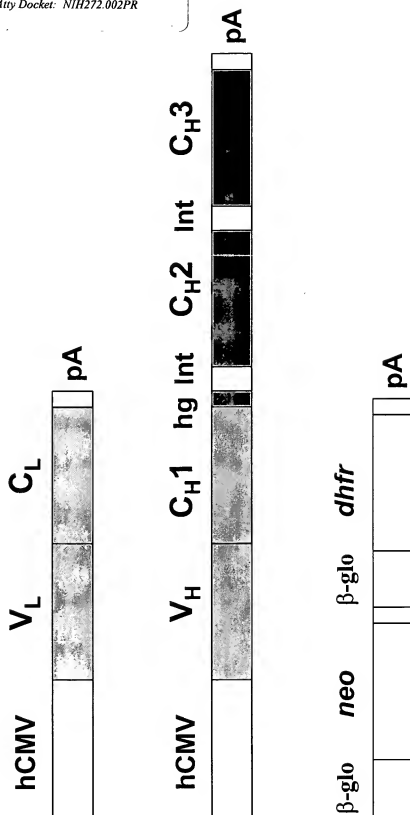


Fig. 2

| (A) | FR1 | | | | FR2 | | | | CDR2 | | | |
|-----|--------------------------------|--|--|--|------------------------|--|--|--|---------------|--|--|--|
| | CDR1 | | | | FR2 | | | | CDR2 | | | |
| 5A7 | --ELTQGPATLSLSPERATLSC | | | | RAGOSLDS-----SLLS | | | | WYQKPGQAPRLMW | | | |
| 3C1 | ELQM,,S,,S,,A,V,D,V,VT, | | | | ,,SED,N,VHSDGNTY,, | | | | K,,SLES | | | |
| 3E4 | --,,S,,LS,PYTL,QP,SI,, | | | | ,,SS,N,VHSDGNTY,, | | | | KV,N,DS | | | |
| 7G4 | ,,,,S,,S,,A,V,D,V,IT, | | | | ,,S,GIS-----W,A | | | | K,,SLES | | | |
| 5H2 | ELQM,,S,,S,,A,V,D,V,IT, | | | | ,,S,DIS-----IR,N | | | | ,,,,LES | | | |
| 5D9 | --,,S,,S,,A,V,D,V,IT, | | | | ,,S,GIS-----NR,N | | | | ,,,,SLSVS | | | |
| (B) | FR3 | | | | CDR3 | | | | FR4 | | | |
| | CDR3 | | | | FR4 | | | | CDR3 | | | |
| 5A7 | GVPAFSGSGGDTFTLSSIQDPFVY | | | | CQHYNDPRT | | | | FQGTGLEIKRT | | | |
| 3C1 | ,,S,,S,,E,,E,,D,,T,, | | | | ,,YQSY,Y,, | | | | ,,P,, | | | |
| 3E4 | ,,D,,A,,K,TRVEA,,VGL,, | | | | ,,V,GVOF,I,, | | | | ,, | | | |
| 7G4 | ,,S,,E,,E,,D,,T,, | | | | ,,YGSY,L,, | | | | ,,P,,VD,, | | | |
| 5H2 | ,,S,,E,,E,,D,,T,, | | | | ,,FNSY,L,, | | | | ,,G,,V,, | | | |
| 5D9 | ,,S,,E,,E,,D,,T,, | | | | ,,FNSY,L,, | | | | ,,G,, | | | |
| (B) | FR1 | | | | CDR1 | | | | FR2 | | | |
| | CDR1 | | | | FR2 | | | | CDR2 | | | |
| 5A7 | EVOLLES-GGGLVOPGGSLRLSCAASGVTF | | | | SYNMH | | | | WVRQAPGKLEW | | | |
| 3C1 | ,,,,,-,AEVKK,,VKV,,KV,,G,L, | | | | ,,GIS | | | | ,,Q,P,,M | | | |
| 3E4 | ,,,,QS,AEVKK,,VKV,,KV,,G,L, | | | | ,,RNPIS | | | | ,,Q,P,,M | | | |
| 7G4 | ,,,,,-,AEVKK,,VKV,,KV,,G,L, | | | | ,,RNPIS | | | | ,,Q,P,,M | | | |
| 5H2 | ,,,,,-,P,,K,SET,S,T,TV,,GSI, | | | | ,,DFYWS | | | | ,,L,S,, | | | |
| 5D9 | ,,,,,-,P,,K,SET,S,T,TV,,GSI, | | | | ,,DFYWS | | | | ,,L,S,, | | | |
| (B) | FR3 | | | | CDR3 | | | | FR4 | | | |
| | CDR3 | | | | FR4 | | | | CDR2 | | | |
| 5A7 | RFTISDRNAKTLYLQMSLRADETAVYIC | | | | SRGGLNWSPRRIETKTPFDY | | | | WQGTGLTVSS | | | |
| 3C1 | ,,V,YTA,ESTS,V,MELS,,S,, | | | | AT-----G,,GRYPTGS,, | | | | ,,A,, | | | |
| 3E4 | ,,V,YTA,ESTS,V,MELS,,S,, | | | | AT-----G,,GRYPTGS,, | | | | ,,A,, | | | |
| 7G4 | ,,,,S,,S,,S,,S,,S,,S,, | | | | T,-----RITTLTVISDA,,I | | | | ,,M,, | | | |
| 5H2 | ,,V,,V,TS,,Q,S,RLSAVT,A,,L,, | | | | A,-----QGTGTTGVSED,F,L | | | | ,,K,I,,L | | | |
| 5D9 | ,,V,,V,TS,,Q,S,RLSAVT,A,,L,, | | | | A,-----QGTGTTGVSED,F,L | | | | ,,K,I,,L | | | |

Fig. 3A

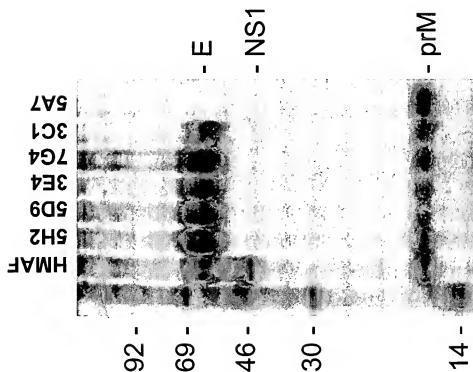


Fig. 3B

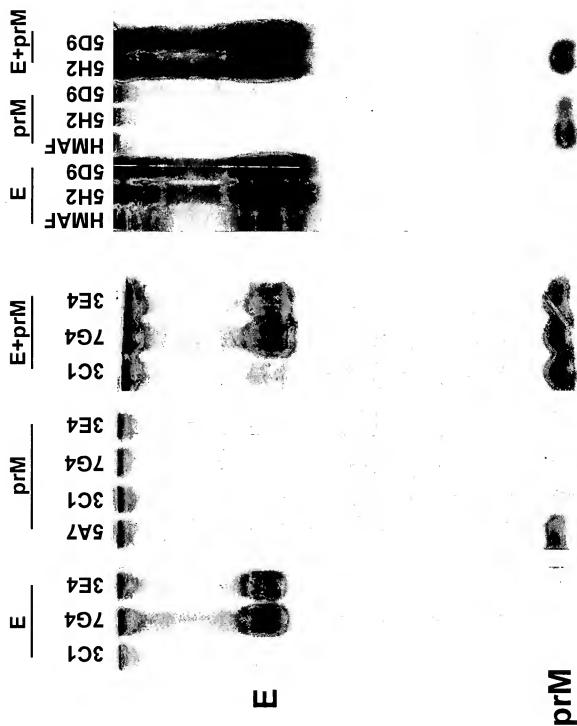


Fig. 4

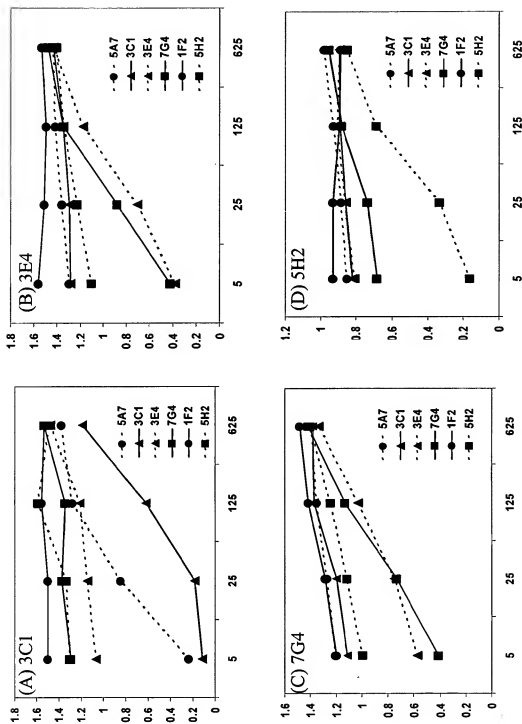


Fig. 5

